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Molecular Brain Research 104 (2002) 240–245

MOLECULAR BRAIN RESEARCH

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Short communication

Effect of *Brn-3a* deficiency on nociceptors and low-threshold mechanoreceptors in the trigeminal ganglion

H. Ichikawa[a,b,*], Z. Mo[c], M. Xiang[c], T. Sugimoto[a,b]

[a]*Department of Oral Function and Anatomy, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8525, Japan*

[b]*Biodental Research Center, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8525, Japan*

[c]*Center for Advanced Biotechnology and Medicine, and Department of Pediatrics, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA*

Accepted 6 June 2002

Abstract

Immunohistochemistry for protein gene product 9.5 (PGP 9.5, a neuron specific protein) and vanilloid receptor 1-like receptor (VRL-1, a marker for medium-sized to large primary nociceptors) were used to assess the effects of *Brn-3a* deficiency on neuronal innervation of oral tissues and neurons of the trigeminal ganglion (TG). In the knockout mouse, the number of PGP 9.5-immunoreactive (-ir) nerve fibers decreased in the facial cutaneous and oral mucous epithelia, as well as the incisor and molar tooth germs. The reduction of PGP 9.5-ir Merkel endings was also observed in some vibrissae. No obvious change was detected in other tissues. Cell size analysis demonstrated that the proportion of small neurons markedly increased while that of medium-sized and large neurons significantly decreased in the TG of the mutant. Moreover, *Brn-3a* deficiency caused the disappearance of TG neurons which were immunoreactive for VRL-1. Together, our data suggest that nociceptors and low-threshold mechanoreceptors with medium-sized to large cell bodies may be sensitive to the loss of *Brn-3a*.

Theme: Sensory systems

Topic: Somatic and visceral afferents

Keywords: *Brn-3a*; Knockout mouse; Protein gene product 9.5; Trigeminal ganglion; Immunohistochemistry; Vanilloid receptor 1-like receptor

1. Introduction

Brn-3a/Brn-3.0, a member of the POU family of transcription factors, is predominantly expressed by neurons [4,6,7,18,24]. This factor induces the expression of *Bcl-2* gene and protects neurons from apoptosis [3,16,18,23]. Brn-3a also activates a number of other neuronally expressed genes and stimulates outgrowth of neuronal processes [16,21,22]. During mouse development, the trigeminal ganglion (TG) shows intense *Brn-3a* expression [25]. Previous studies have demonstrated that targeted deletion of *Brn-3a* gene in mice results in a marked reduction of neurons in the TG [2,7,25]. In the *Brn-3a* mutants, only 30% of the normal complement of neurons survive till birth [7]. The surviving neurons are devoid of Trk receptors, but approximately 70% of them express the glial cell derived neurotrophic factor receptor [7]. These findings suggest a developmental dependency of Trk-positive neurons on Brn-3a in the TG.

Trigeminal primary neurons innervate a wide variety of tissues in the oro-facial region. These include the facial skin, lip, tongue and palate. Previous studies have classified the primary sensory neurons into several subpopulations based on their morphology [9,12,15,20]. Nociceptive neurons are small to medium-sized in the TG [15,20]. They supply the cutaneous and mucosal epithelia with free nerve endings [20]. In addition, the tooth pulp has been considered to be innervated exclusively by nociceptive afferents in the TG. Such neurons mostly have large cell bodies [8,13]. Recently, we investigated cutaneous and

*Corresponding author. Tel.: +81-86-235-6636; fax: +81-86-235-6612.

E-mail address: hiroichi@md.okayama-u.ac.jp (H. Ichikawa).

0169-328X/02/$ – see front matter
PII: S0169-328X(02)00355-8

pulpal TG neurons for vanilloid receptor 1-like receptor (VRL-1), a marker for medium-sized to large nociceptors [1,13]. VRL-1-positive TG neurons were abundant in the tooth pulp but rare in the facial skin [13]. On the other hand, low-threshold mechanoreceptors are large and innervate Merkel endings and corpuscular endings in the oro-facial structures [9–12]. During normal development, the survival of nociceptive and mechanoreceptive primary sensory neurons is thought to depend on Trks in the TG [5,10,11,14,17].

In the present study, the distribution of protein gene product 9.5 (PGP 9.5, a neuron specific protein) [5,10,17] was examined in the oro-facial tissues of wildtype and *Brn-3a* knockout mice to understand the effect of *Brn-3a* deficiency on different types of TG neurons. The cell size of TG neurons was also investigated in the mutant mouse. In addition, immunohistochemistry for VRL-1 was performed in the TG.

2. Materials and methods

Mice lacking the *Brn-3a* gene were prepared as described by Xiang et al. [25]. Four wildtype and four knockout mice at postnatal day 0 (P0) were obtained from mating of *Brn-3a* heterozygous mice. Animals were immersion-fixed overnight in 4% paraformaldehyde at 4 °C. Subsequently, tissues were stored at 4 °C in phosphate-buffered saline (PBS) containing 0.1 mM sodium azide until use. The head containing the TG, facial skin and vibrissal pad as well as the tongue, palate, lip and tooth was dissected, immersed in PBS containing 20% sucrose overnight, frozen-sectioned sagittally at 10 μm, and thaw-mounted on gelatin-coated glass slides. Nissl and immunohistochemical stains were performed in these sections. For immunohistochemistry, the sections were incubated with rabbit anti-PGP 9.5 serum (1:15 000, UltraClone) or rabbit anti-VRL-1 serum [1] followed by the incubation with biotinylated goat anti-rabbit IgG and ABC-complex (Vector Laboratories). The number of VRL-1-ir neurons was counted in every tenth of the serial sections of the TG. For cell size analysis, the microscopic images of Nissl- or VRL-1-stained cell bodies were projected over a digitizer tablet using a drawing tube (×215). The cross-sectional area of those cell bodies that contained the nucleolus was recorded.

The specificity of the primary antisera used in this study has been described elsewhere [1,10].

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.

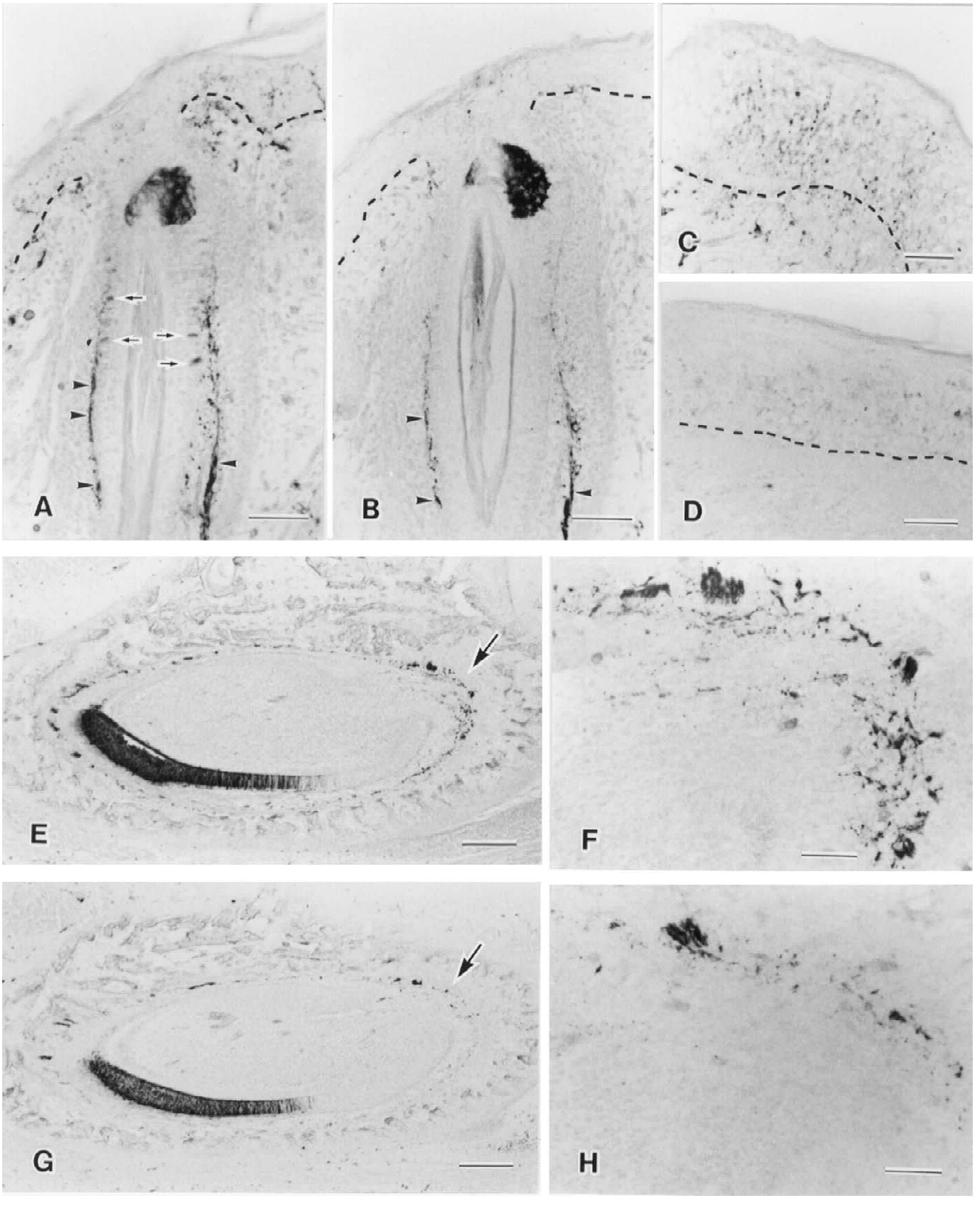
3. Results

3.1. PGP 9.5-ir nerve fibers in peripheral tissues

At P0, the oro-facial tissues contained numerous PGP 9.5-ir nerve fibers in wildtype mice. In the vibrissal pad, facial skin, lip, tongue and hard palate, PGP 9.5-ir nerve fibers formed subepithelial nerve plexuses (Fig. 1A, C). They occasionally penetrated the cutaneous and mucosal epithelia, and terminated as varicose endings within the epithelia (Fig. 1A, C). These fibers were abundant in the labial mucosa (Fig. 1C) and rare in the tongue. In the vibrissal pad and facial skin, PGP 9.5-ir nerve fibers ran to the base of the hair, and sent their thick neurites in parallel with the hair shaft (Fig. 1A). In the vibrissa, PGP 9.5-ir varicose fibers were also seen near PGP 9.5-ir Merkel cells (Fig. 1A). In the tongue and soft palate, numerous PGP 9.5-ir fibers made nerve plexuses beneath the taste bud (data not shown). Many PGP 9.5-ir nerve fibers were also observed within the taste bud of the fungiform papilla and soft palate. Distinct corpuscular endings could not be observed in the vibrissal pad, facial skin or oral mucosa. In the lacrimal gland and the major and minor salivary glands, PGP 9.5-ir nerve fibers were detected around secretory acini and ducts. In the musculature of the head, abundant motor end plates were immunoreactive for PGP 9.5 (data not shown). The muscle spindle also contained PGP 9.5-ir spiral nerve endings. In the incisor, many PGP 9.5-ir nerve fibers were seen around the tooth germ. Numerous varicose fibers were concentrated near the root apex of the incisor (Fig. 1F). In the molar, however, only a few PGP 9.5-ir nerve fibers surrounded the tooth germ (data not shown).

In *Brn-3a* null mutant mice, remarkable changes in the distribution of PGP 9.5-ir nerve fibers were detected in the vibrissal pad, skin, mucosa and tooth germ (Fig. 1B, D, G, H). The number of PGP 9.5-ir nerve fibers markedly decreased in and beneath the epithelium of the vibrissal

Fig. 1. Immunohistochemical microphotographs of PGP 9.5 in the vibrissal pad, labial mucosa and incisor tooth germ of wildtype (A, C, E, F) and *Brn-3a* knockout (B, D, G, H) mice at postnatal day 0. *Brn-3a* deficiency causes a marked reduction in the number of PGP 9.5-ir nerve fibers in the cutaneous and mucosal epithelia (A–D). Dotted lines in A–D show the border between the epithelium and the lamina propria. The number of Merkel endings (arrows in A) also decreases in the knockout mouse. Arrowheads point to thick neurites which run parallel to the hair shaft in wildtype (A) and knockout mice (B). The number of ir nerve fibers surrounding the incisor tooth germ greatly decreases in the mutant mouse (E, G). The reduction is notable near the root apex (arrows in E and G). Panels F and H are at a higher magnification than panels E and G, respectively. Bars=100 μm (A, B), 50 μm (C, D, F, H) and 200 μm (E, G).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pad (Figs. 1B), facial skin and labial mucosa (Figs. 1D). However, the distribution of PGP 9.5-ir nerve fibers was similar in the lingual epithelium of wildtype and mutant mice (figure not shown). In some vibrissal follicles of the knockout mouse, the number of PGP 9.5-ir Merkel cells and varicose fibers also showed a significant decrease (Fig. 1B). Around the tooth germ, PGP 9.5-ir nerve fibers similarly decreased in number (Fig. 1G, H). The reduction was more conspicuous in the incisor tooth germ than in the molar tooth germ. No obvious change was observed in other tissues such as the guard hair, taste bud, lacrimal and salivary glands or musculature.

3.2. The trigeminal ganglion

At P0, the TG contained abundant neurons in the wildtype but a greatly reduced number of neurons in the knockout mouse (Fig. 2A, B). Cell size analysis showed that TG neurons were of various sizes in wildtype mice (mean±S.D.=168.5±91.4 μm^2, range=24.1–606.6 μm^2, n=585) (Fig. 3). 23.9% (140/585) of them were smaller than 100 μm^2; 44.8% (262/585) measured from 100 to 200 μm^2; and more than 30% had cell bodies >200 μm^2 (31.3% or 183/585). In the knockout mice, however, most TG neurons were small to medium-sized (mean±S.D.= 119.8±78.6 μm^2, range=21.4–487.8 μm^2, n=454) (Fig. 3). More than a half (51.8% or 235/454) of them were smaller than 100 μm^2; 34.1% (155/454) fell in the range of 100–200 μm^2; and TG neurons >200 μm^2 were relatively rare in the knockout mouse (14.1% or 64/454).

The TG contained VRL-1-ir neurons in the wildtype mouse (Fig. 2C). The mean number of VRL-1-ir TG neurons per section was 5.1 (S.D.=2.7, n=37). These neurons were medium-sized to large (mean±S.D.= 267.8±74.9 μm^2, range=106.1–471.0 μm^2, n=155). No VRL-1-ir neuron was smaller than 100 μm^2; only 18.7% (29/155) of VRL-1-ir TG neurons fell in the range of 100–200 μm^2; and the large majority of them measured

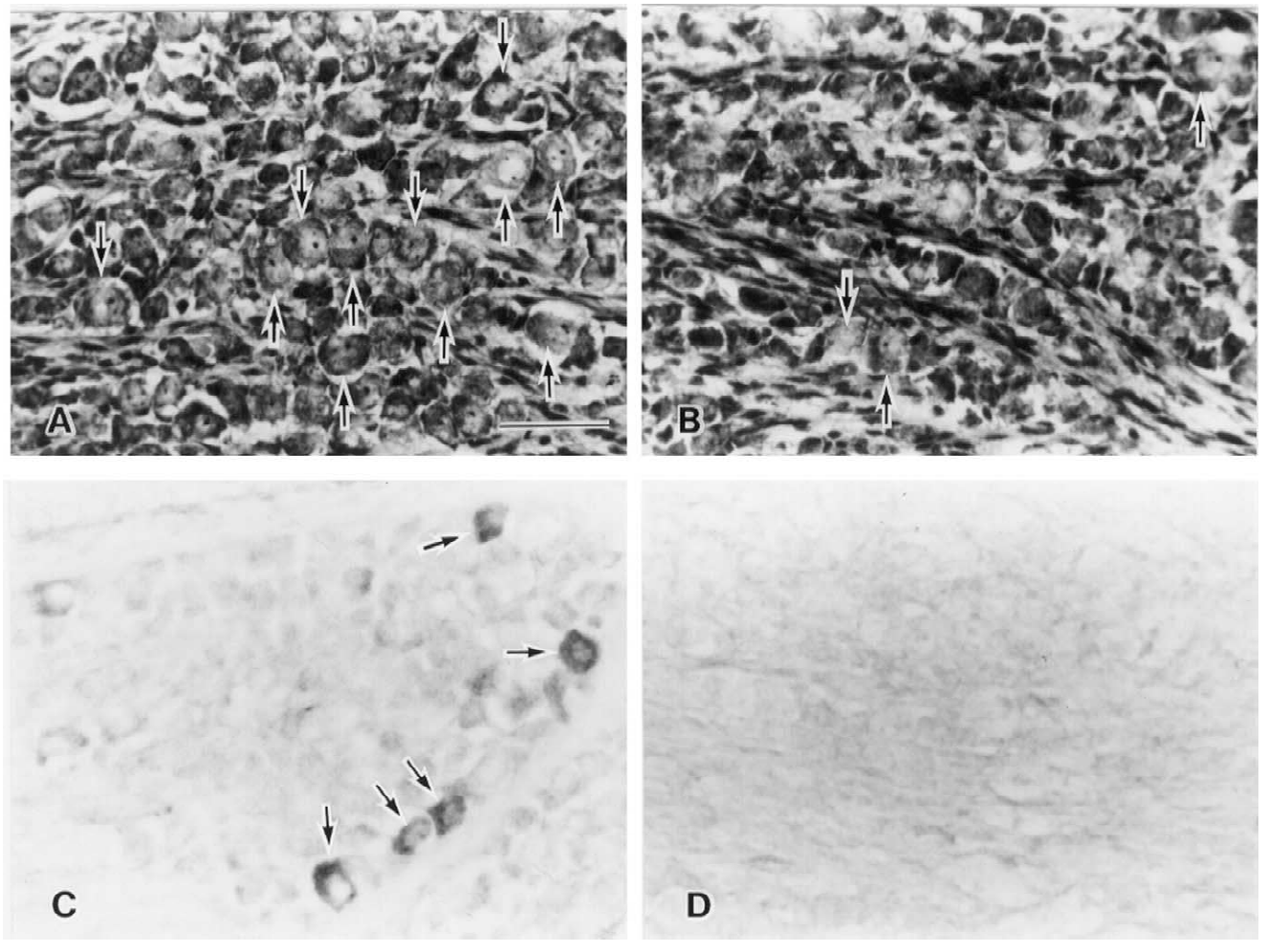

Fig. 2. Microphotographs of Nissl-stained TG (A, B) or TG labeled with the anti-VRL-1 antibody (C, D) from P0 wildtype (A, C) and *Brn-3a* knockout (B, D) mice. Large TG neurons are abundant in the wildtype mouse (arrows in A) but rare in the knockout mouse (arrows in B). The TG contains VRL-1-ir neurons in the wildtype mouse (arrows in C) but not the mutant mouse (D). Bar=100 μm (A). All figures are at the same magnification.

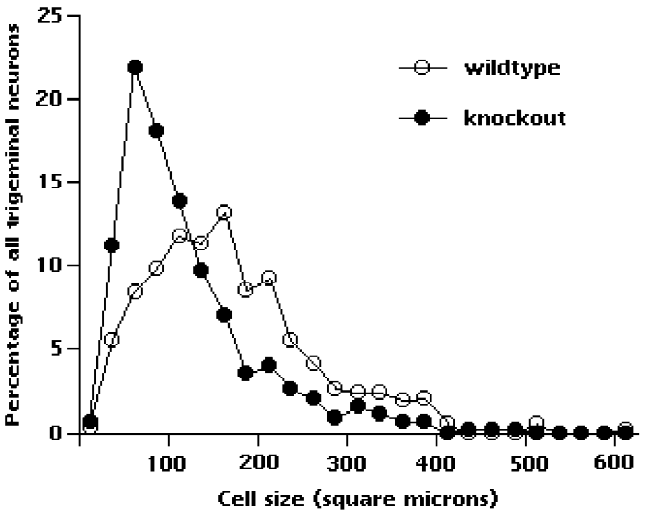

Fig. 3. Cell size spectra of TG neurons in wildtype and *Brn-3a* knockout mice at P0. The data were obtained from 585 and 454 neurons in wildtype and knockout mice, respectively.

larger than 200 μm^2 (81.3% or 126/155). In the knockout mouse, however, the TG was devoid of any VRL-1-ir neurons (Fig. 2D).

4. Discussion

In the present study, we showed the distribution of PGP 9.5-ir nerve fibers in oro-facial tissues of wildtype mice. In the vibrissal pad, facial skin and oral mucosa, the epithelium had abundant PGP 9.5-ir nerve endings. Vibrissae and hairs were also innervated by PGP 9.5-ir nerve fibers. In addition, PGP 9.5-ir nerve fibers were observed around secretory ducts in the lacrimal and salivary glands. The incisor and molar tooth germs were surrounded by the ir nerve fibers as well. Because PGP 9.5-ir is detected in neurons of the superior cervical, pterygopalatine, intralingual and submandibular ganglia (our own unpublished data), the possibility that such nerve fibers are derived from the autonomic ganglia cannot be ruled out. However, their distribution and morphology suggest that these nerve fibers are mostly sensory and therefore originate from the TG.

Our immunohistochemical analysis of wildtype and knockout mice demonstrated that *Brn-3a* deficiency dramatically reduced the number of PGP 9.5-ir nerve fibers in the skin, mucosa and tooth germ. Within the facial cutaneous epithelium and oral mucous epithelium, PGP 9.5-ir nerve fibers mostly disappeared. The number of PGP 9.5-ir nerve fibers also significantly decreased in incisor and molar tooth germs. These findings suggest that the loss of *Brn-3a* may cause the reduction of nociceptive fibers in oro-facial structures. Interestingly, we found that the proportion of small TG neurons increased but that of medium-sized and large TG neurons decreased in the knockout mice. Together with the previous observation that 70% of TG neurons were lost in the mutant by P0 [7], our data suggest that *Brn-3a* deficiency may reduce the number of facial and oral nociceptors with medium-sized and large cell bodies. This idea is supported by the present finding that VRL-1-ir TG neurons, which are presumed to be medium-sized to large nociceptors [1,13], are entirely missing in the mutant mouse.

In some vibrissae of the knockout mouse, we found that the number of PGP 9.5-ir Merkel cells and varicose fibers significantly decreased. Together with the previous report that denervation in newborn rats caused a marked reduction of Merkel cells [19], our data suggest that the decrease of Merkel cells may be due to the loss of primary sensory neurons innervating these cells. It is likely that low-threshold mechanoreceptors which innervate vibrissal Merkel cells are sensitive to *Brn-3a* deficiency. This notion can be supported by our finding that the proportion of large neurons is greatly reduced in the mutant TG.

In conclusion, the present study investigated the effect of *Brn-3a* deficiency on PGP 9.5-ir nerve fibers in oro-facial tissues. The loss of *Brn-3a* significantly reduced the number of ir nerve fibers in the facial cutaneous and oral mucous epithelia, as well as the incisor and molar tooth germs. The reduction of PGP 9.5-ir Merkel endings was also observed in some vibrissae. Correspondingly, in the TG of the knockout mouse, the proportion of small neurons greatly increased while that of medium-sized and large neurons significantly decreased. In addition, VRL-1-ir TG neurons completely disappeared in the mutant. Thus, nociceptors and low-threshold mechanoreceptors with medium-sized to large cell bodies in the TG may be sensitive to the loss of *Brn-3a*.

Acknowledgements

We greatly appreciate Dr David Julius at the Department of Cellular and Molecular Pharmacology, University of California for his kind gift of the anti-VRL-1-serum.

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