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Effect of *Brn-3a* deficiency on primary nociceptors in the trigeminal ganglion

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Abstract

Immunohistochemistry for substance P, somatostatin and vanilloid receptor subtype 1 as well as receptors for somatostatin and opioids was performed on the trigeminal ganglion in wild-type and *Brn-3a* knockout mice at postnatal day 0. In wild-type mice, the trigeminal ganglion contained abundant substance P-, vanilloid receptor subtype 1-, sst2A receptor- and delta-opioid receptor-immunoreactive neurons, while the ganglion had only a few mu-opioid receptor-immunoreactive neurons. The *Brn-3a* deficiency had an effect on the cell size but not the number of substance P-immunoreactive neurons. In knockout mice, the proportion of small immunoreactive neurons markedly increased and that of medium- to large-sized immunoreactive ones correspondingly decreased (mean ± S.D. = 54.7 ± 29.1 μm^2, range = 10.9–220.8 μm^2) compared to wild-type mice (mean ± S.D. = 116.6 ± 58.6 μm^2, range = 27.3–400.7 μm^2). As for vanilloid receptor subtype 1-immunoreactive neurons, the number and cell size was barely affected by the deficiency. On the other hand, the loss of *Brn-3a* caused a decrease in the number of sst2A receptor- or delta-opioid receptor-immunoreactive neurons (more than 95% reduction) and an increase in the number of mu-opioid receptor-immunoreactive neurons (9.3-fold increase). Somatostatin-immunoreactive neurons were not detected in the trigeminal ganglion of wild-type or mutant mice at postnatal day 0.

The present study suggests that *Brn-3a* deficiency may have effects on the survival of trigeminal nociceptors and their expression of some neurochemical substances.

Keywords: Transcription factor; Knockout mouse; Neuropeptides; Capsaicin receptor; Trigeminal ganglion; Immunohistochemistry

1. Introduction

Previous studies have demonstrated that primary nociceptors contain several neurochemical substances in the trigeminal ganglion (TG). Substance P (SP) and somatostatin (SOM) are markers for small primary sensory neurons (Goedert et al., 1984; Kai-Kai, 1989; Ambalavanar and Morris, 1992). Calcitonin gene-related peptide (CGRP) is localized to small- and medium-sized neurons (Skofitsch and Jacobowitz, 1985). These neurons supply their peripheral receptive fields with free nerve endings, and are considered to be primary nociceptors in the ganglion (Goedert et al., 1984; Ishida-Yamamoto et al., 1989; Silverman and Kruger, 1989). Double immunofluorescence methods have revealed that small CGRP-IR neurons also express SP or SOM (Gibbins et al., 1987; Quartu et al., 1992). The capsaicin (vanilloid) receptor VR1, which can be activated by vanilloid compounds, protons and heat (>43 °C), is detected in small- to medium-sized neurons in the TG (Caterina et al., 1997). These neurons co-express CGRP and SP, and send their unmyelinated axons to orofacial tissues (Caterina et al., 1997; Ichikawa and Sugimoto, 2001; Ichikawa et al., 2004). As well, the receptors for SOM

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and opioids have been demonstrated in the ganglion; sst2A and mu-opioid receptors (MOR) are localized to small- or medium-sized neurons and co-expressed by CGRP in these neurons (Li et al., 1998; Ichikawa et al., 2003). The sst2A receptor- and MOR-containing neurons supply the nasal mucosa or tooth pulp with their unmyelinated axons (Taddese et al., 1995; Ichikawa et al., 2003). In addition, the mRNA for delta-opioid receptor (DOR) is detected in the TG but its cellular distribution has never been reported in the ganglion (Buzas and Cox, 1997).

Brn-3a/Brn-3.0 is a member of the POU family of transcription factors which is predominantly expressed by neurons (Gerrero et al., 1993; Fedtsova and Turner, 1995; Xiang et al., 1995; McEvilly et al., 1996; Huang et al., 2001). This factor induces the expression of *bcl-2* gene and protects neurons from apoptosis (McEvilly et al., 1996; Latchman, 1998; Smith et al., 1998; Ensor et al., 2001). In addition, Brn-3a activates a number of other neuronally expressed genes and stimulates outgrowth of neuronal processes (Smith et al., 1997a, 1997b; Latchman, 1998). During the mouse development, *Brn-3a* is highly expressed by the trigeminal ganglion (TG) (Xiang et al., 1996). Targeted deletion of *Brn-3a* gene results in a marked reduction in the number of TG neurons (McEvilly et al., 1996; Xiang et al., 1996; Huang et al., 1999). Recently, we have investigated CGRP-IR TG neurons in Brn-3a knockout mice (Ichikawa et al., 2002b). Although the total number of CGRP-IR TG neurons is similar in wild-type and knockout mice, the *Brn-3a* deficiency causes a decrease in the number of medium-sized IR neurons and an increase in the number of small IR neurons. This suggests that Brn-3a deficiency may affect the cell body size and neurochemical content of primary nociceptors. However, little is known about the effect of the deficiency on primary nociceptors other than CGRP-IR ones.

In the present study, the distribution in the TG of SP, SOM and VR1 as well as receptors for SOM and opioids was compared between wild-type and *Brn-3a* knockout mice.

2. Materials and methods

Mice lacking the *Brn-3a* gene were prepared as described by Xiang et al. (1996). Five wild-type and five knockout mice at postnatal day 0 were obtained from breeding 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 including the TG, vibrissa, facial skin, palate and lip was dissected, immersed in PBS containing 20% sucrose overnight, frozen-sectioned sagittally at 10 μm, and thaw-mounted on gelatin-coated glass slides. Complete series of sections were divided into 10 subsets so that every tenth sections were mounted on the same slides. Each subseries was stained for one of the 6 antigens to be examined. Sections were incubated overnight with rabbit anti-SP serum (1:50,000, Peninsula, USA), rabbit anti-SOM serum (1:50,000, Peninsula), rabbit anti-VR1 serum (1:50,000, Neuromics, USA), rabbit anti-sst-2A serum (1:30,000, Schulz et al., 1998b), rabbit anti-DOR serum (1:50,000) [27] or rabbit anti-MOR serum (1:30,000, Schulz et al., 1998a) followed by incubation with biotinylated goat anti-rabbit IgG and avidin-biotin-horseradish peroxidase complex (Vector Laboratories, USA). The number of IR neurons was counted in all sections of the subseries. For cell size analysis, the microscopic image (×215) of IR cell bodies was projected over a digitizer tablet using a drawing tube. The cross-sectional area of those cell bodies that contained the nucleolus in some representative sections was recorded.

The specificity of the primary antisera used in this study has been described elsewhere (Ichikawa et al., 1989; Schulz et al., 1998a, 1998b; Ichikawa and Sugimoto, 2001).

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. Neuropeptides

In wild-type mice, the TG contained abundant SP-IR neurons (Fig. 1A), and their number was barely affected by the *Brn-3a* deletion (Table 1). These IR neurons showed no topographic preference in both wild-type and mutant mice. In the knockout mice, however, medium- and large-sized SP-IR neurons (cell body size $> 100\ \mu m^2$) mostly disappeared (Fig. 1B). Cell size analysis demonstrated that SP-IR neurons in wild-type mice were of various sizes (Fig. 2). Half (47.3% or 243/514) of them were small ($<100\ \mu m^2$), 43% (221/514) were medium-sized (100–200 μm^2) and

Table 1
Counts of immunoreactive neurons in the trigeminal ganglion of wild-type and knockout mice at P0

	Wild type ($n = 4$)	Knockout ($n = 4$)
Substance P	1111.5 ± 97.3	1202.8 ± 41.4
Vanilloid receptor subtype 1	176.8 ± 41.0	179.3 ± 24.3
sst2A receptor	931.8 ± 139.0	96.5 ± 42.6*
Delta-opioid receptor	105 ± 25.8	5.8 ± 1.9*
Mu-opioid receptor	30.3 ± 14.7	283.0 ± 19.1*

Values represents mean ± S.E. Difference between wild-type and knockout mice was significant (*$P < 5\%$, Mann–Whitney). The data were obtained from four wild-type and mutant mice.

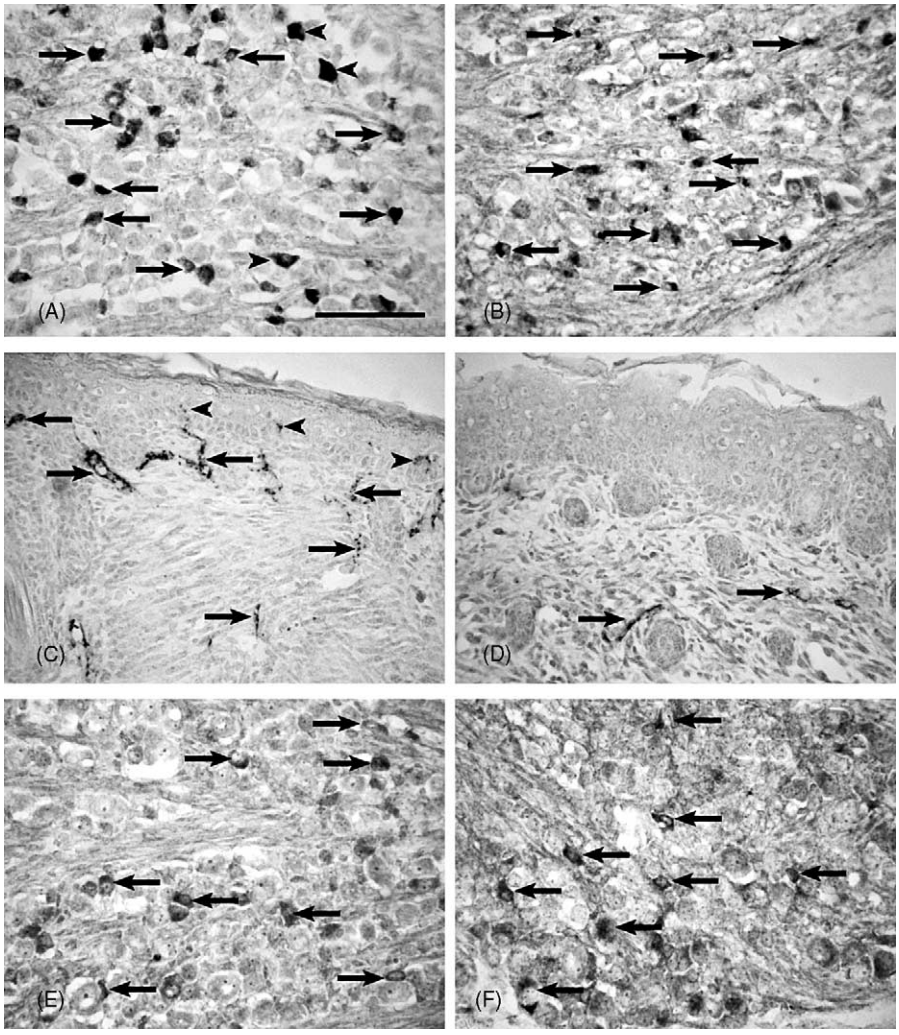

Fig. 1. Immunohistochemical microphotographs of SP-IR (A, B, C, D) and VR1-IR (E, F) in the TG (A, B, E, F) and facial skin (C, D) of wild-type (A, C, E) and *Brn-3a* knockout (B, D, F) mice at P0. SP-IR TG neurons are small (arrows in A) or large (arrowheads in A) in the wild-type mouse, whereas such neurons are mostly small in the knockout mouse (arrows in B). Brn-3a deficiency causes a marked reduction in the number of cutaneous SP-IR nerve fibers (arrows in C, D). Arrowheads in C point to intraepithelial IR endings. On the other hand, the distribution and cell size of VR1-IR TG neurons are similar in wild type (arrows in E) and mutant (arrows in F). Bar = 100 μm (A). All figures are at the same magnification.

about 10% (50/514) were large (>200 μm^2) (Fig. 2). In knockout animals the proportion of small SP-IR neurons showed a 2-fold increase (93.5% or 491/525), while that of medium-sized ones showed an 80% decrease (6.3% or 33/525) (Fig. 2). Large SP-IR neurons almost disappeared in the mutants (0.2% or 1/525).

In wild-type mice, many SP-IR fibers and endings were seen in the oro-facial region (Fig. 1C). The infraorbital and inferior alveolar nerves contained many SP-IR nerve fibers. The vibrissal pad, facial skin and lip of the wild-type mouse contained abundant SP-IR endings (see Fig. 1C for example in the skin). These endings were detected in the vibrissal follicle, epithelium and subepithelial connective tissue. Brn-3a deficiency significantly decreased the number of such endings. For instance, SP-IR intraepithelial nerve endings mostly disappeared in the knockout mouse (Fig. 1D).

The TG and peripheral tissues were devoid of SOM-IR in both wild-type and knockout mice at P0 (data not shown).

3.2. Capsaicin receptor

Many VR-1-IR neurons were observed throughout the TG in wild-type and knockout mice (Figs. 1E, F). The number and cell size spectrum of IR neurons were similar in these animals (Table 1, Fig. 3). Half of VR1-IR neurons were small in wild-type (49.1% or 107/218) and knockout mice (54.5% or 116/213), and more than 40% were medium-sized (wild type: 105/218, knockout: 89/213). The TG was mostly devoid of large VR1-IR neurons (wild type: 2.8% or 6/218, knockout: 3.8% or 8/213). VR1-IR nerve fibers could not be detected in the TG or oro-facial region.

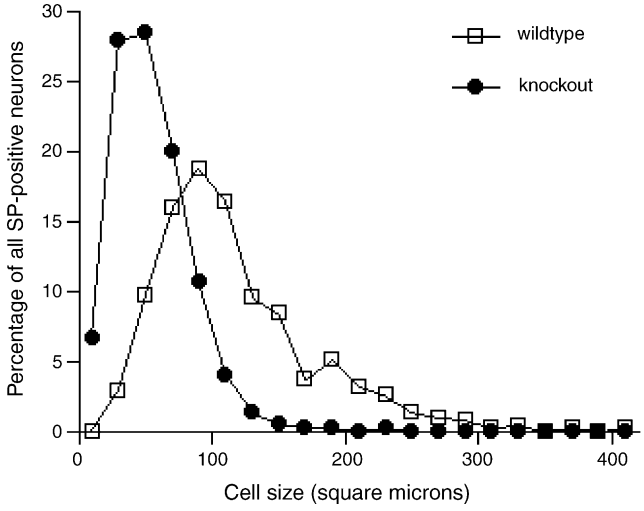

Fig. 2. Cell size spectra of SP-IR TG neurons in P0 wild-type and *Brn-3a* knockout mice. The mean ± S.D. of SP-IR cell sizes in wild-type and knockout mice was 116.6 ± 58.6 μm^2 (range = 27.3–400.7 μm^2, n = 514) and 54.7 ± 29.1 μm^2 (range = 10.9–220.8 μm^2, n = 525), respectively.

3.3. Neuropeptide receptors

The sst2A receptor-IR neurons were abundant throughout the TG in wild-type mice (Fig. 4A). The IR was localized to the cytoplasmic membrane. Their axons were also immunoreactive for the receptor protein within the TG. The absence of Brn-3a dramatically decreased the number of IR neurons (97% reduction, Table 1, Fig. 4B). Cell size analysis indicated that wild-type sst2A receptor-IR neurons had predominantly medium- (67.9% or 152/224) or large-sized (25% or 56/224) cell bodies (Fig. 5). In mutant mice, medium- and large-sized IR neurons were markedly reduced. As a result, the proportion of small IR neurons increased from 7.1% (16/224, wild type) to 40.5% (36/89, knockout) (Fig. 5). sst2A-IR nerve fibers could not be seen in oro-facial tissues of wild-type or knockout mice.

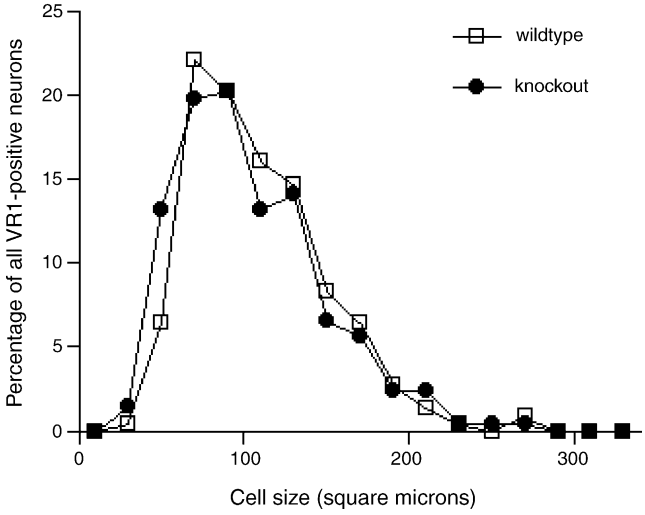

Fig. 3. Cell size spectra of VR1-IR TG neurons in P0 wild-type and *Brn-3a* knockout mice. The mean ± S.D. of VR1-IR cell sizes in wild-type and knockout mice was 108.9 ± 41.8 μm^2 (range = 35.9–262.2 μm^2, n = 218) and 104.9 ± 43.4 μm^2 (range = 34.6–261.4 μm^2, n = 213), respectively.

Numerous DOR-IR neurons were observed throughout the wild-type TG (Fig. 4C). The IR was localized to the cytoplasm but not the nucleus of these neurons. The *Brn-3a* disruption caused a great decrease in the number of such neurons (95% reduction Table 1, Fig. 4D). Cell size analysis indicated that wild-type DOR-IR neurons were mostly small- (56.4% or 119/211) or medium-sized (42.7% or 90/211) (Fig. 6). Only 1% (2/211) had large cell bodies. In mutant mice, small- and medium-sized DOR-IR decreased and the proportion of large IR neurons significantly increased (13.6% or 3/22, Fig. 6). DOR-IR nerve fibers could not be detected in the TG or oro-facial region of both animals.

The wild-type TG contained a small number of MOR-IR neurons (Table 1). The IR was localized to the cytoplasmic membrane. The absence of *Brn-3a* dramatically increased the number of MOR-IR neurons (9.3-fold increase, Table 1, Fig. 4E and F). In addition, numerous MOR-IR nerve fibers appeared within the TG (Fig. 4F). Cell size spectra of MOR-IR neurons were similar in wild-type and knockout mice; i.e., they were mostly small-sized (wild type: 62.3% or 38/61, knockout: 73.9% or 153/207) or medium-sized (wild type: 34.4% or 21/61, knockout: 23.7% or 49/207) (Fig. 7). Large MOR-IR neurons were very rare in wild-type (3.3% or 2/61) and mutant (2.4% or 5/207) mice. MOR-IR nerve fibers could not be detected in the oro-facial region of wild-type mice. In the mutant, numerous IR nerve fibers appeared within the infraorbital and inferior alveolar nerves. However, their endings could not be detected in the vibrissal pad, facial skin, lip or palate.

4. Discussion

Primary nociceptors have small- to medium-sized cell bodies in the TG. They supply the cutaneous and mucosal epithelia with free nerve endings (Goedert et al., 1984; Silverman and Kruger, 1989). During normal development, these neurons express TrkA, a high-affinity receptor for nerve growth factor (NGF), and their survival is thought to depend on the neurotrophic substance (Klein, 1994; Fundin et al., 1997). Compared to wild-type mice, only 30% of TG neurons can survive in Brn-3a knockout mice (Huang et al., 1999). The disruption also results in a loss of TrkA-expressing neurons in the TG (Huang et al., 1999). Moreover, intraepithelial nerve fibers decrease in the facial skin and oral mucosa of the knockout mouse (Ichikawa et al., 2002a). Together with the fact that Brn-3a can promote the survival of NGF-dependent sensory neurons (McEvilly et al., 1996), the evidence suggests that the survival of primary nociceptors is dependent upon Brn-3a in the TG.

The present study investigated the effect of *Brn-3a* deficiency on primary nocicepors and their neurochemical expression in the TG. In knockout mice, the number of sst2A

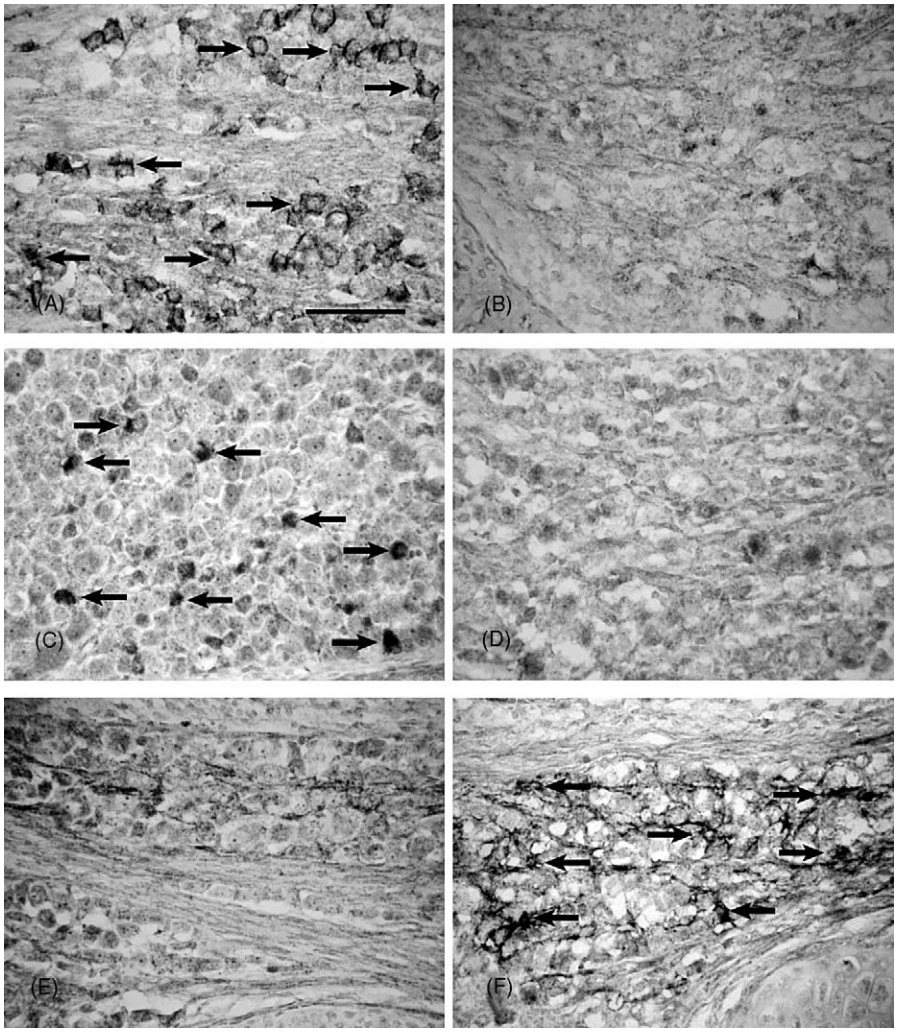

Fig. 4. Immunohistochemical microphotographs of sst2A receptor-IR (A, B), DOR-IR (C, D) and MOR-IR (E, F) in the TG of wild-type (A, C, E) and *Brn-3a* knockout (B, D, F) mice at P0. The number of sst2A receptor- and DOR-IR TG neurons markedly decreases in the knockout mouse (B, D) compared to the wild-type mouse (arrows in A, C). The absence of *Brn-3a* causes an increase in the number of MOR-IR neurons (E, arrows in F). Bar = 100 μm (A). All figures are at the same magnification.

receptor- and DOR-IR neurons greatly decreased. Therefore, it is likely that their survival depends on Brn-3a in this ganglion. Unlike these neurons, the loss of *Brn-3a* caused a dramatic increase in the number of MOR-IR neurons. Many small- to medium-sized MOR-IR cells appeared in the TG of knockout mice. Correspondingly, MOR-IR nerve fibers appeared in the infraorbital and inferior alveolar nerves. The effect of Brn-3a deficiency on large MOR-IR remains unclear in this study. It is unlikely that the survival of MOR-IR TG neurons or their MOR expression depends on NGF. In Brn-3a knockout mice, their development is probably dependent upon other substances such as glial cell line-derived neurotrophic factor (Huang et al., 1999). Selective loss of TG neurons induced by Brn-3a knockout might have reduced a possible competition for the chemical substances required for survival of MOR-IR neurons. It is also possible that the loss of Brn-3a has an effect on the proliferation of MOR-IR neurons and/or their MOR expression in the TG.

In *Brn-3a* knockout mice, no obvious change was observed in the number of SP-IR neurons. However, the cell size spectra of SP-IR TG neurons were quite different between wild-type and knockout mice. The proportion of medium- and large-sized SP-IR neurons greatly decreased while that of small SP-IR neurons markedly increased in the knockout mouse. The change of SP-IR cell size spectrum was similar to that of CGRP-IR cell size spectrum which has been previously reported in the mutant (Ichikawa et al., 2002b). *Brn-3a* deficiency probably causes an increase in the number of small SP-IR neurons and a decrease in the number of larger SP-IR neurons. In the epithelium of knockout mice, the number of SP-IR nerve fibers and endings decreased as well. A previous study demonstrated that Brn-3a deficiency caused defects in the normal axonal growth and target innervation of sensory neurons which precede sensory neural death in the knockout mouse (Eng et al., 2001). Therefore, it is likely that the axon growth of SP-IR neurons

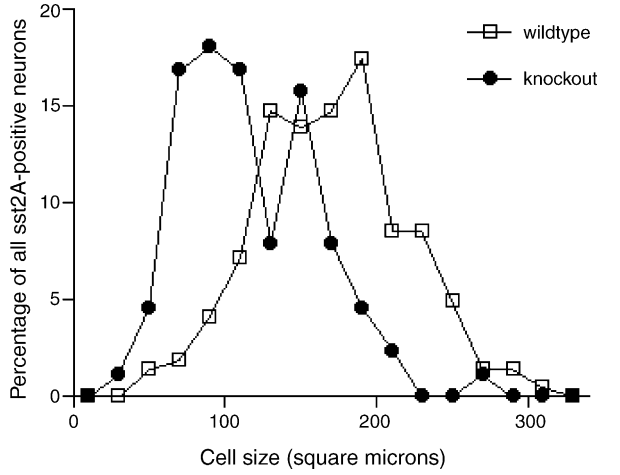

Fig. 5. Cell size spectra of sst2A receptor-IR TG neurons in P0 *Brn-3a* wild-type and knockout mice. The mean ± S.D. of sst2A receptor-IR cell sizes in wild-type and knockout mice was 170.2 ± 48.4 μm^2 (range = 52.2–313.3 μm^2, n = 224) and 122.0 ± 50.4 μm^2 (range = 37.5–305.4 μm^2, n = 89), respectively.

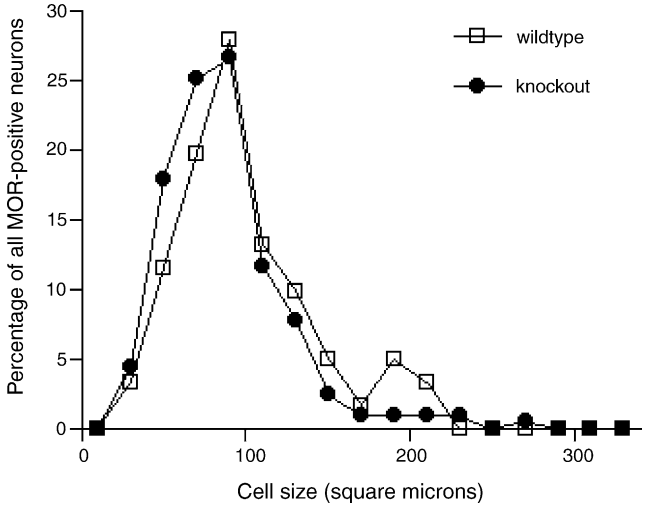

Fig. 7. Cell size spectra of MOR-IR TG neurons in P0 wild-type and *Brn-3a* knockout mice. The mean ± S.D. of MOR-IR cell sizes in wild-type and knockout mice was 99.1 ± 42.1 μm^2 (range = 37.7–218.4 μm^2, n = 61) and 87.0 ± 37.2 μm^2 (range = 29.9–262.9 μm^2, n = 207), respectively.

which supply the cutaneous and mucosal innervation depend on Brn-3a in the TG. Alternatively, Brn-3a may be associated with the proliferation of small SP-IR TG neurons and/or their expression of SP. Unlike in wildtype mice, the survival of SP-IR neurons is considered to be independent upon NGF and TrkA in knockout mice. Further studies will be necessary to understand the mechanism of their developmental dependency on neurotrophic substances. On the other hand, the similarity in distribution of VR1-IR neurons among wild-type and knockout mice suggests that Brn-3a deficiency has little or no effect on their survival or VR1 expression. In this study, however, it remains unclear whether or not VR1-IR neurons co-express SP-IR in knockout mice. The content of VR1 in peripheral axons may be too low to be detected by the present immunohistochemical method, because VR1-IR nerve fibers could not be observed in oro-facial regions.

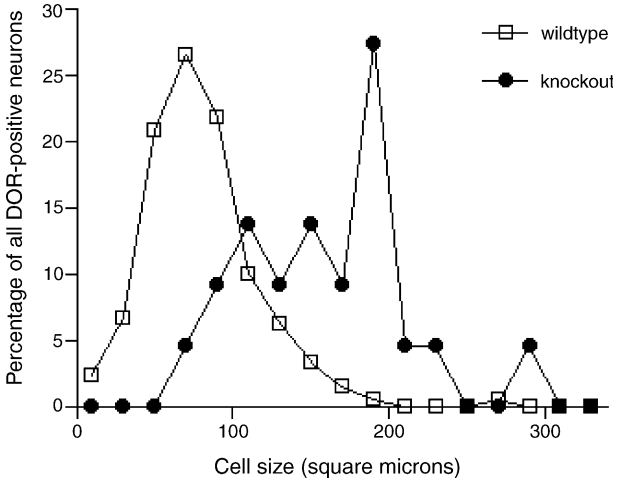

Fig. 6. Cell size spectra of DOR-IR TG neurons in P0 wild-type and *Brn-3a* knockout mice. The mean ± S.D. of DOR-IR cell sizes in wild-type and knockout mice was 100.4 ± 35.1 μm^2 (range = 23.0–286.2 μm^2, n = 211) and 158.0 ± 51.7 μm^2 (range = 67.4–287.2 μm^2, n = 22), respectively.

In conclusion, the present study investigated the effect of *Brn-3a* deficiency on primary nociceptors in the TG. The loss of *Brn-3a* reduced the number of the sst2A receptor- and DOR-IR neurons. By contrast, the number of MOR-IR neurons dramatically increased in knockout mice. In addition, lack of *Brn-3a* function exerted differential effects on small and larger SP-IR TG neurons. These data together suggest that *Brn-3a* deficiency may have effects on the survival and proliferation of trigeminal nociceptors and/or their expression of some neurochemical substances.

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