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EFFECT OF *Brn-3a* DEFICIENCY ON PARVALBUMIN-, CALBINDIN D-28k-, CALRETININ- AND CALCITONIN GENE-RELATED PEPTIDE-IMMUNOREACTIVE PRIMARY SENSORY NEURONS IN THE TRIGEMINAL GANGLION

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Abstract—Immunohistochemistry for parvalbumin, calbindin D-28k, calretinin and calcitonin gene-related peptide (CGRP) was performed on the trigeminal ganglion and oro-facial tissues in *Brn-3a* wildtype and knockout mice at embryonic day 18.5 and postnatal day 0. In wildtype mice, the trigeminal ganglion contained abundant parvalbumin-, calbindin D-28k- and CGRP-immunoreactive neurons while the ganglion was almost devoid of calretinin-immunoreactive neurons. In *Brn-3a* knockout mice, a 63% decrease of parvalbumin-immunoreactive neurons was detected. In contrast, the absence of *Brn-3a* dramatically increased the number of calbindin D-28k-immunoreactive (3.5-fold increase) and calretinin-immunoreactive neurons (91-fold increase). The number of CGRP-immunoreactive neurons, however, was not altered by the *Brn-3a* deficiency. Cell size analysis indicated that loss *of Brn-3a* increased the proportions of small (<100 μm^2) parvalbumin-, calbindin D-28k- and CGRP-immunoreactive neurons while it decreased those of large (>200 μm^2) immunoreactive cells. Calretinin-immunoreactive neurons were either small or medium (100–200 μm^2) in mutant mice. The oro-facial tissues contained parvalbumin-, calbindin D-28k- and CGRP-immunoreactive fibers, but not calretinin-immunoreactive ones in wildtype mice. In *Brn-3a* knockout mice, the number of parvalbumin-immunoreactive fibers markedly decreased in the infraorbital nerve and parvalbumin-immunoreactive endings disappeared in the vibrissa. In contrast, the number of calbindin D-28k-immunoreactive fibers increased significantly in the infraorbital and mental nerves. In addition, calbindin D-28k-immunoreactive endings appeared in the vibrissa. As well, some fibers showed calretinin-immunoreactivity in the infraorbital nerve of the mutant. However, no obvious change of CGRP-immunoreactive fibers was observed in the oro-facial region of knockout mice.

Taken together, our data suggest that *Brn-3a* deficiency has effects on the expression of neurochemical substances in the trigeminal ganglion.

Key words: transcription factor, knockout mouse, neurochemical substances, primary sensory neurons, immunohistochemistry.

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 the *bcl-2* gene and protects neurons from apoptosis (McEvilly et al., 1996; Latchman, 1998; Smith et al., 1998; Ensor et al., 2001). Brn-3a also activates a number of other neuronally expressed genes and stimulates outgrowth of neuronal processes (Smith et al., 1997a,b; Latchman, 1998). During the mouse development, the trigeminal ganglion (TG) shows intense *Brn-3a* expression (Xiang et al., 1996). Previous studies have demonstrated that targeted deletion of the *Brn-3a* gene in mice results in a marked reduction of neurons in the TG (McEvilly et al., 1996; Xiang et al., 1996; Huang et al., 1999). In the *Brn-3a* mutants, only 30% of the normal complement of neurons survive until birth (Huang et al., 1999). Because of the absence of Trk receptors in the surviving neurons, Brn-3a is considered to control the survival and differentiation of trigeminal neurons by regulating expression of the neurotrophin receptors (Huang et al., 1999).

Previous immunohistochemical studies have classified primary sensory neurons into several subpopulations on the basis of their chemical markers. Parvalbumin, calbindin D-28k and calretinin, members of the calcium-binding protein family, are predominantly localized to large

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Abbreviations: CGRP, calcitonin gene-related peptide; E, embryonic day; ION, infraorbital nerve; -IR, -immunoreactive or -immunoreactivity; MN, mental nerve; P, postnatal day; PBS, phosphate-buffered saline; TG, trigeminal ganglion.

neuronal cell bodies in the TG (Celio, 1990; Ichikawa et al., 1993, 1994, 1996a, 1997). Such neurons innervate corpuscular endings in the oro-facial region and are considered to be low-threshold mechanoreceptors (Ichikawa et al., 1996b, 2000; Ichikawa and Sugimoto, 1997). Calcitonin gene-related peptide (CGRP) is considered to be a marker specific to small to medium-sized neurons in the TG (Skofitsch and Jacobowitz, 1985). CGRP-immunoreactive (-IR) primary sensory neurons supply their peripheral receptive field with free nerve endings, and are thought to participate in nociception (Ishida-Yamamoto et al., 1989). During normal development, the survival of mechanoreceptive and nociceptive primary sensory neurons is thought to depend on Trk receptors in the TG (Klein, 1994; Minichiello et al., 1995; Fundin et al., 1997; Ichikawa et al., 2000, 2001).

In the present study, the distribution of parvalbumin-, calbindin D-28k-, calretinin- and CGRP-IR neurons was examined in the TG of *Brn-3a* wildtype and knockout mice. The innervation of these neurons was also investigated in oro-facial tissues.

EXPERIMENTAL PROCEDURES

Mice lacking the *Brn-3a* gene were derived as described by Xiang et al. (1996). Two wildtype and four knockout mice at embryonic day 18.5 (E18.5) and five wildtype and six knockout mice at postnatal day 0 (P0) 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 containing the TG, vibrissa, 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. These sections were incubated overnight with rabbit anti-parvalbumin serum (1:50 000, Swant, Switzerland), rabbit anti-calbindin D-28k serum (1:50 000, Swant), rabbit anti-calretinin serum (1:50 000; Winsky et al., 1989) or rabbit anti-CGRP serum (1:50 000, Peninsula Laboratories, Belmont, CA, USA), followed by incubation with biotinylated goat anti-rabbit IgG and avidin–biotin–horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The number of immunoreactive neurons was counted in every tenth section of contiguous sections. For cell size analysis, the microscopic image (×215) of immunoreactive cell bodies was projected over a digitizer tablet using a drawing tube. 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 (Ichikawa et al., 1989, 1996a,b; Winsky et al., 1989).

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.

All data are presented as mean ± S.D.

RESULTS

Trigeminal ganglion

In wildtype mice, the TG contained abundant parvalbumin-, calbindin D-28k- and CGRP-IR neurons (Figs. 1A, 1C, 2C) while the ganglion was almost devoid of calretinin-IR neurons (Fig. 2A). Topographically, the distribution of parvalbumin-IR neurons in the TG was not uniform, i.e., they were more concentrated in the rostral half of the ganglion (192.3 ± 36.8 immunoreactive neurons) than in the caudal half (66 ± 19.3 immunoreactive neurons). At P0, a 63% decrease of parvalbumin-IR neurons was detected in mutant mice (Table 1, Fig. 1B). This reduction was primarily due to the decrease in the rostral half of the TG (49.3 ± 29.6 immunoreactive neurons, 74% decrease). In the caudal half the difference between the wildtype and mutant (47.3 ± 13.5 immunoreactive neurons) was minute and statistically insignificant. However, the absence of *Brn-3a* dramatically increased the number of calbindin D-28k-IR (3.6-fold increase) and calretinin-IR neurons (91-fold increase) (Table 1, Figs. 1C, D, 2A, B). Unlike parvalbumin-IR neurons, these immunoreactive neurons showed no topographic preference in both wildtype and mutant mice. CGRP-IR neurons were numerous throughout the ganglion, and their number was barely affected by the *Brn-3a* deletion (Table 1). In contrast, large CGRP-IR neurons (cell body size >200 μm^2) almost completely disappeared in the mutants (see below).

Cell size analysis indicated that P0 wildtype parvalbumin-IR neurons had medium-sized (100–200 μm^2, 48%) or large (>200 μm^2, 50.4%) cell bodies (mean ± S.D. = 205.7 ± 62.4 μm^2, range = 80.7–455.3 μm^2, $n = 244$) (Fig. 3). In mutant mice the proportion of large parvalbumin-IR neurons slightly decreased (38.5%) and that of small ones (<100 μm^2) increased from 1.64% (wildtype) to 12.8%. The mean ± S.D. and the distribution range of parvalbumin-IR cell sizes in mutant mice were 179.5 ± 69.4 μm^2 and 71.1–379.2 μm^2, respectively ($n = 117$) (Fig. 3). Cell size spectrum of P0 wildtype calbindin D-28k-IR neurons (Figs. 1C, 4) was similar to that of parvalbumin-IR neurons, i.e., they were mostly medium-

Table 1. Counts of immunoreactive neurons in the trigeminal ganglion of wildtype and knockout mice at P0

	Wildtype	Knockout
Parvalbumin	258.3 ± 45.5 ($n = 4$)	96.5 ± 42.6* ($n = 4$)
Calbindin D-28k	238.3 ± 37.5 ($n = 4$)	857.8 ± 98.5* ($n = 4$)
Calretinin	2.8 ± 3.1 ($n = 4$)	251.5 ± 19.5* ($n = 4$)
CGRP	881 ± 279.2 ($n = 4$)	787.8 ± 87.9 ($n = 4$)

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

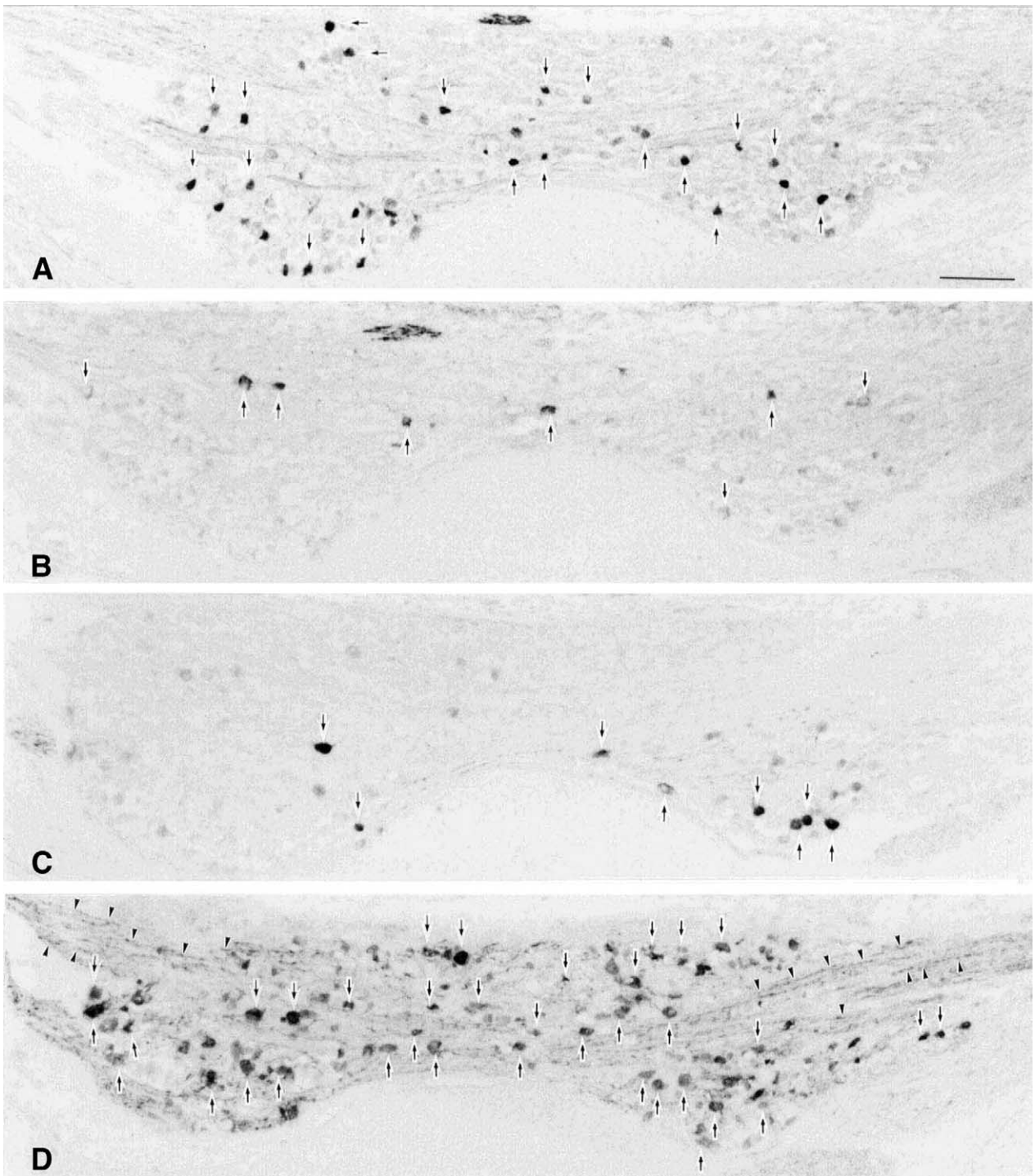

Fig. 1. Immunohistochemical microphotographs of parvalbumin- (A, B) and calbindin D-28k-IR (C, D) in the TG of *Brn-3a* wildtype (A, C) and knockout (B, D) mice at P0. *Brn-3a* deficiency causes a marked reduction in the number of parvalbumin-IR TG neurons (arrows in A, B). In contrast, the number of calbindin D-28k-IR TG neurons (arrows) and their axons (arrowheads) greatly increases in the mutant (D) compared to the wildtype (C). Scale bar = 100 μm.

sized (31.2%) or large (62.3%) (mean ± S.D. = 233.8 ± 89.7 μm^2, range = 37.2–555.2 μm^2, $n = 413$). The proportion of small cells was dramatically increased by the *Brn-3a* disruption from 6.5% (wildtype) to 43.6%, while the proportions of medium-sized and large cells decreased accordingly (mean ± S.D. = 147.6 ± 105.3 μm^2, range = 18.2–591.4 μm^2, $n = 440$). Calretinin-IR neurons were extremely rare (nine immunoreactive cells in a total of eight sections examined at P0) and thus cell size analysis of these neurons was not performed in wildtype mice. However, we observed many calretinin-IR cells in P0 mutant mice (mean ± S.D. = 88.1 ± 36.0 μm^2, range = 22.0–192.9 μm^2, $n = 208$). They were either small (67.3%) or medium (32.7%) and no calretinin-IR neurons larger

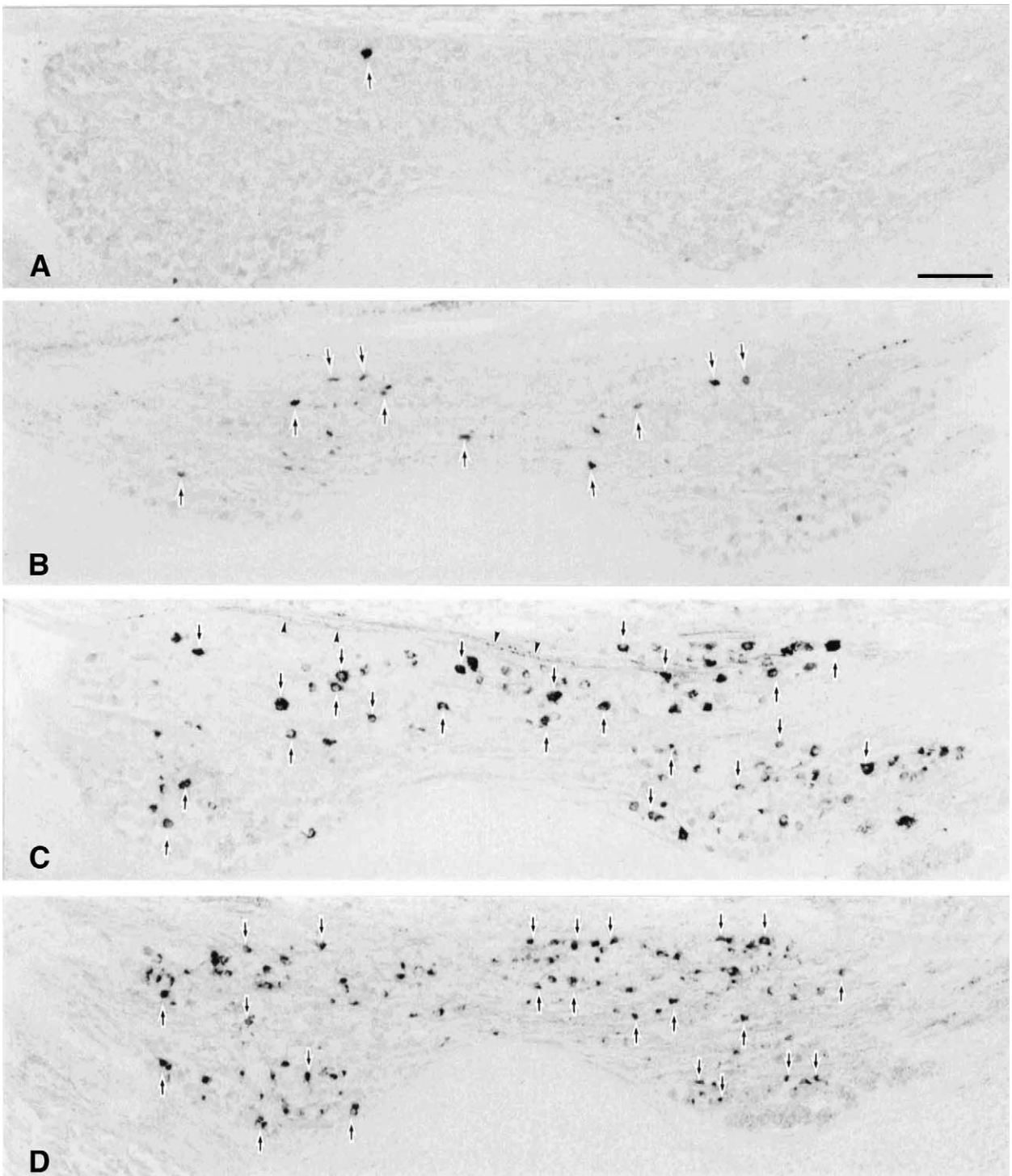

Fig. 2. Immunohistochemical microphotographs of calretinin- (A, B) and CGRP-IR (C, D) in the TG of *Brn-3a* wildtype (A, C) and knockout (B, D) mice at P0. The number of calretinin-IR TG neurons greatly increases in the mutant (arrows in B) compared to the wildtype (an arrow in A). CGRP-IR TG neurons are of various sizes in the wildtype mouse (arrows in C), whereas such neurons are mostly small in the knockout mouse (arrows in D). Scale bar = 100 μm.

than 200 μm^2 was observed. CGRP-IR neurons in P0 wildtype mice were of various sizes (mean ± S.D. = 161.0 ± 73.0 μm^2, range = 25.9–412.6 μm^2, $n = 414$) (Figs. 2C, 5). In P0 mutants the proportion of small CGRP-IR neurons showed a three-fold increase, while that of medium-sized ones showed a 60% decrease. Large CGRP-IR neurons, which made up 25.4% of all CGRP-IR neurons in the wildtype, almost completely disappeared in the mutants. The mean ± S.D. and the distribution range of CGRP-IR cell sizes in mutant mice were 78.3 ± 44.2 μm^2 and 15.0–280.8 μm^2, respectively ($n = 410$).

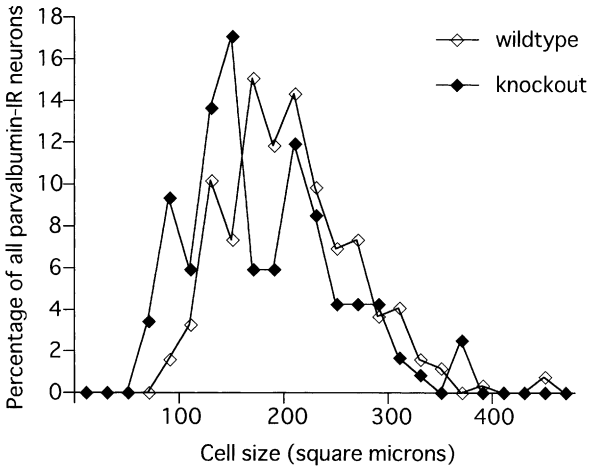

Fig. 3. Cell size spectra of parvalbumin-IR TG neurons in P0 *Brn-3a* wildtype and knockout mice. The data were obtained from 244 and 117 neurons in wildtype and knockout mice, respectively.

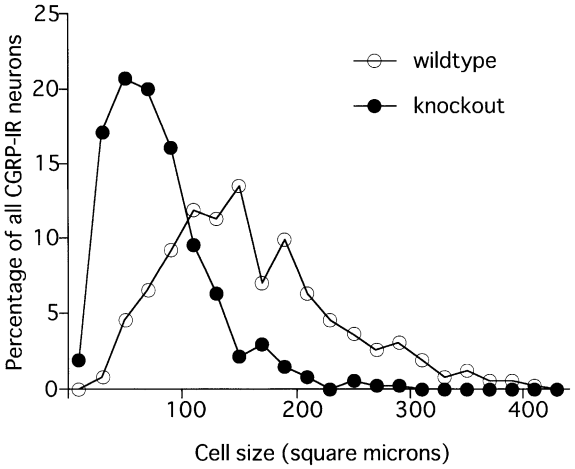

Fig. 5. Cell size spectra of CGRP-IR TG neurons in P0 *Brn-3a* wildtype and knockout mice. The data were obtained from 414 and 410 neurons in wildtype and knockout mice, respectively.

Peripheral tissues

The oro-facial region contained parvalbumin-, calbindin D-28k-, calretinin- and CGRP-IR fibers and endings in both *Brn-3a* wildtype and knockout mice. In wildtype mice at E18.5 and P0, the infraorbital nerve (ION) contained parvalbumin-, calbindin D-28k- and CGRP-IR fibers but not calretinin-IR fibers (Fig. 6A, C, E, G). Numerous fibers exhibited parvalbumin-IR, and a much smaller number showed calbindin D-28k- and CGRP-IR (Fig. 6A, C, G). In the ION of E18.5 and P0 knockout mice, however, most of the parvalbumin-IR fibers disappeared (Fig. 6B). In contrast, the number of calbindin D-28k-IR fibers greatly increased in the mutant mice (Fig. 6D). In addition, a small but substantial number of calretinin-IR fibers appeared in the mutant mice (Fig. 6F). The number of CGRP-IR fibers, however, displayed no obvious change in the ION of the knockout mouse (Fig. 6H).

In the mental nerve (MN) of E18.5 and P0 wildtype mice, although only a few parvalbumin-, calbindin D-28k- and calretinin-IR fibers were observed (Fig. 7A, C), the CGRP-IR fibers were abundant (Fig. 7E). In *Brn-3a* knockout mice, the number of calbindin D-28k-IR fibers markedly increased and numerous calbindin D-28k-IR fibers were detected in nerve bundles (Fig. 7D). By contrast, no obvious change was observed for parvalbumin-, calretinin- and CGRP-IR fibers in the MN of knockout mice (Fig. 7B, F).

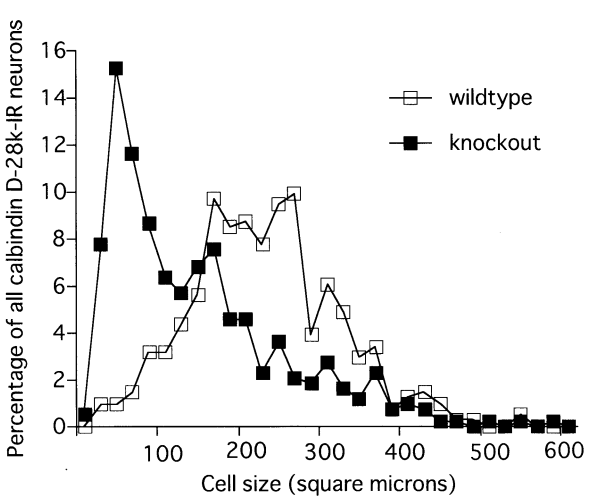

Fig. 4. Cell size spectra of calbindin D-28k-IR TG neurons in P0 *Brn-3a* wildtype and knockout mice. The data were obtained from 413 and 440 neurons in wildtype and knockout mice, respectively.

The vibrissal pad, facial skin, lip and palate also contained parvalbumin-, calbindin D-28k-, and CGRP-IR nerve endings in E18.5 and P0 wildtype mice. Parvalbumin-IR endings were observed in the vibrissa but not other regions. In the vibrissal pad, parvalbumin-IR nerve fibers ran to the base of vibrissae and sent their varicose terminals to vibrissal follicles (Fig. 8A). Calbindin D-28k-IR endings were detected only in the palate of wildtype mice. In the hard palate, calbindin D-28k-IR thick fibers ran toward the epithelium and made corpuscular endings at the top of palatal rugae (Fig. 8G). These endings appeared to be oval at a low power magnification. At higher power magnifications, however, they had some parallel arrays of calbindin D-28k-IR fibers. A few intraepithelial fibers at the top of palatal rugae also showed calbindin D-28k-IR (Fig. 8G). The oro-facial tissues in wildtype mice were devoid of calretinin-IR endings. The vibrissal pad, facial skin and lip of the wildtype mouse contained abundant CGRP-IR endings (Fig. 8E). These endings were detected in the vibrissal follicle, epithelium and subepithelial connective tissue. CGRP-IR varicose fibers were also observed around the incisor and molar tooth germ in wildtype mice.

In *Brn-3a* knockout mice, the number of parvalbumin-IR fibers markedly decreased in the vibrissal pad and only a few parvalbumin-IR endings were detected in vibrissal follicles (Fig. 8B). However, numerous calbindin D-28k-IR fibers appeared in the vibrissal pad and numerous immunoreactive varicose endings innervated vibrissal follicles (Fig. 8D). In the hard palate, calbindin D-28k-IR intraepithelial endings disappeared while the distribution and morphology of calbindin D-28k-IR cor-

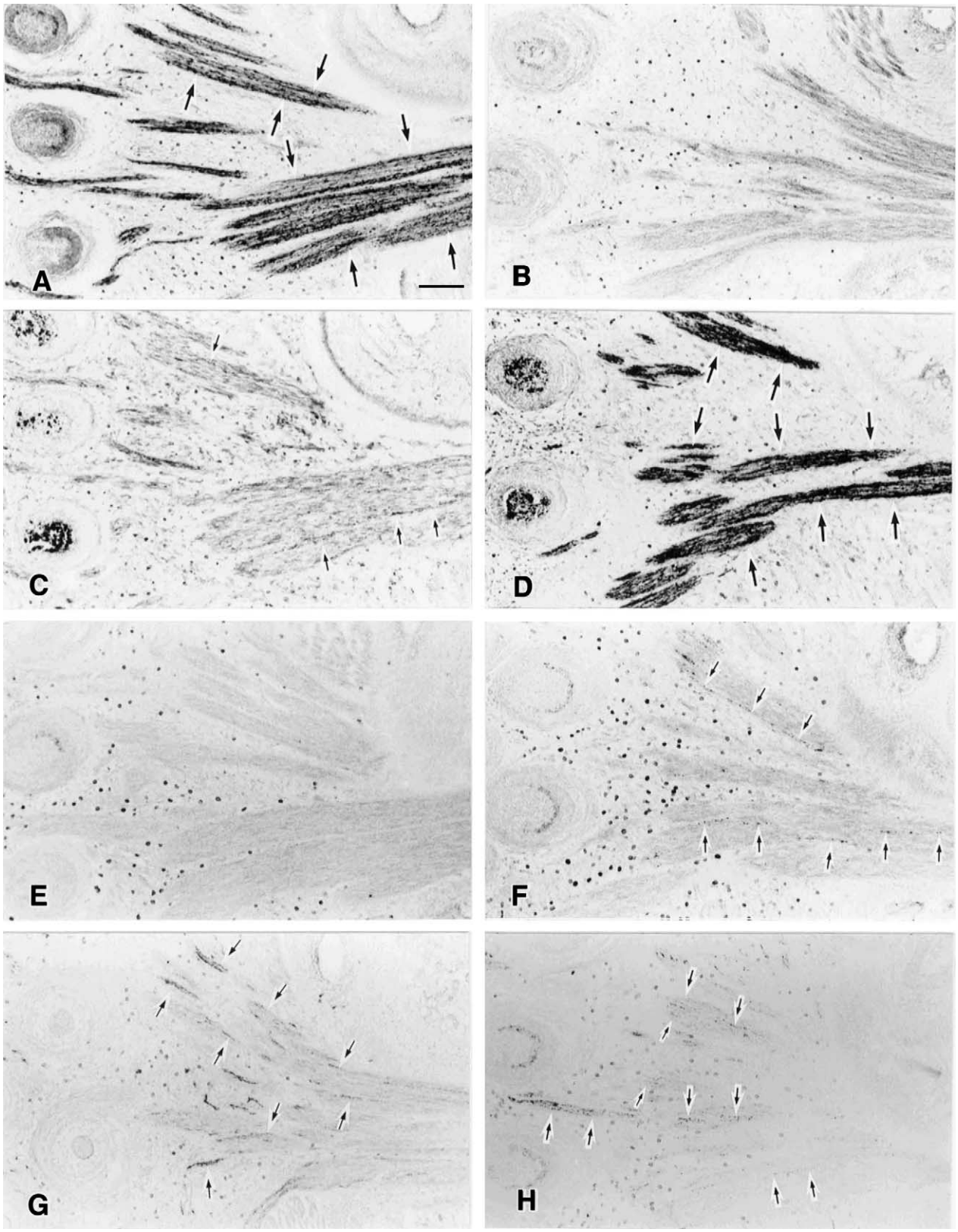

Fig. 6. Immunohistochemical microphotographs of parvalbumin- (A, B), calbindin D-28k- (C, D), calretinin- (E, F) and CGRP-IR (G, H) in the infraorbital nerve (ION) of *Brn-3a* wildtype (A, C, E, G) and knockout (B, D, F, H) mice at E18.5 (A-D, G, H) and P0 (E, F). In the ION of the wildtype mouse, numerous fibers exhibit parvalbumin-IR (arrows in A) and some fibers show calbindin D-28k- (arrows in C) and CGRP-IR (arrows in G). The ION is devoid of calretinin-IR fibers (E). In the ION of the knockout mouse, most of the parvalbumin-IR fibers have disappeared (B). In contrast, the number of calbindin D-28k-IR fibers greatly increases in the mutant mouse (arrows in D). In addition, some calretinin-IR fibers appear in the mutant (arrows in F). No obvious change of CGRP-IR fibers is detected in the ION of the knockout mouse (arrows in H). Scale bar = 100 μm.

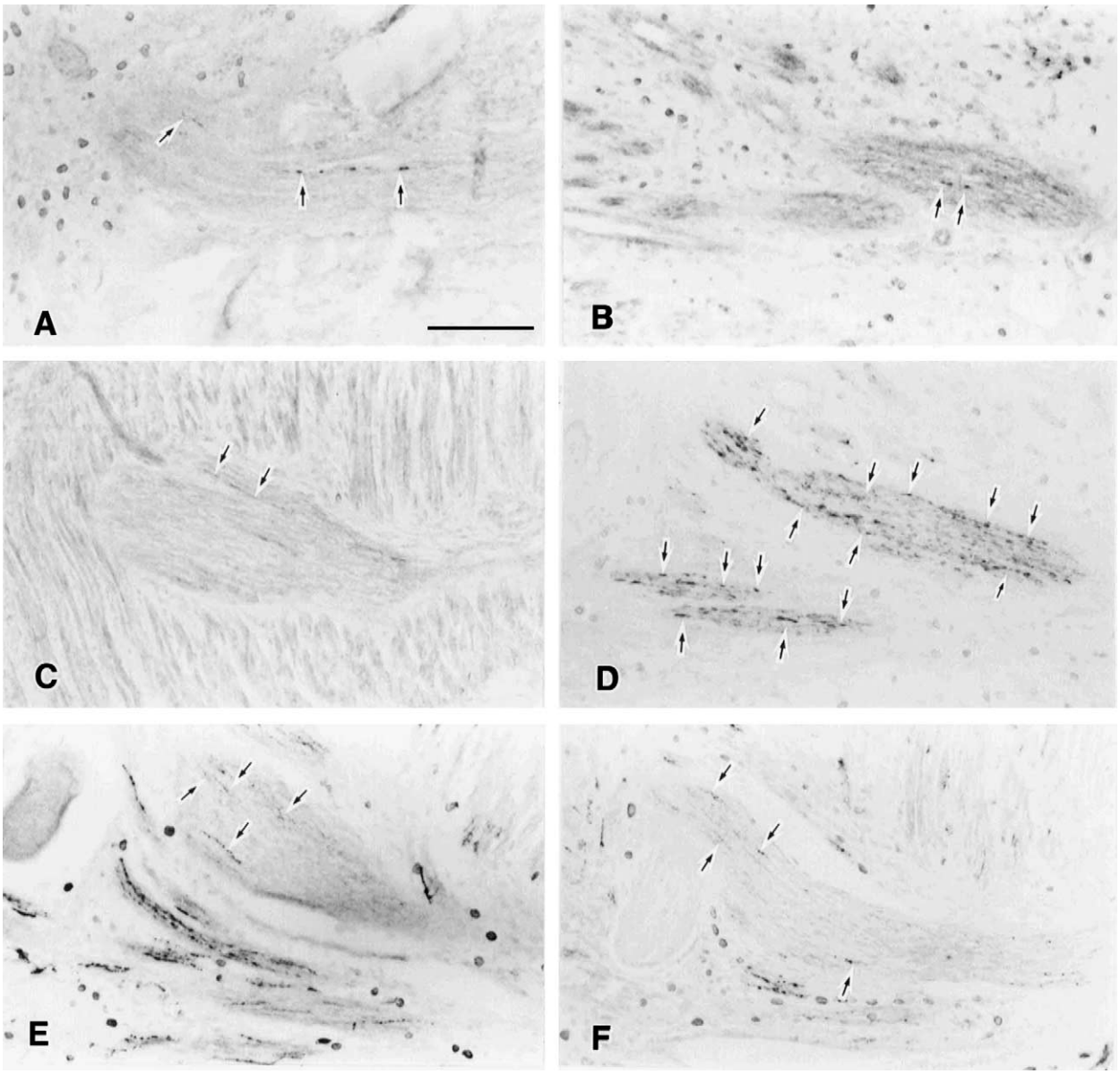

Fig. 7. Immunohistochemical microphotographs of parvalbumin- (A, B), calbindin D-28k- (C, D) and CGRP-IR (E, F) in the mental nerve (MN) of *Brn-3a* wildtype (A, C, E) and knockout (B, D, F) mice at E18.5 (A–D) and P0 (E, F). The MN contains a few parvalbumin-IR fibers in wildtype and knockout mice (arrows in A, B). The number of calbindin D-28k-IR fibers markedly increases in the knockout mouse (arrows in D) compared to the wildtype mouse (arrows in C). No obvious change in the number of CGRP-IR fibers is detected in the MN of the knockout mouse (arrows in E, F). Scale bar = 100 μm.

puscular endings remained unchanged (Fig. 8H). Similar to wildtype mice, no calretinin-IR ending was observed in *Brn-3a* knockout mice. The distribution of CGRP-IR fibers and endings was also similar in wildtype and mutant mice (Fig. 8F).

DISCUSSION

The present study demonstrated the distribution of parvalbumin-, calbindin D-28k-, calretinin- and CGRP-IR in the TG and oro-facial tissues of *Brn-3a* wildtype and knockout mice. The immunoreactivity was detected in cell bodies of primary sensory neurons in the TG. In the oro-facial region, the ION, MN, vibrissal pad, facial skin, lip and palate contained immunoreactive fibers. In addition, immunoreactive nerve endings were detected in the vibrissal follicle, epithelium and subepithelial connective tissue. In this study, the possibility that these immunoreactive fibers and endings are derived from autonomic ganglia cannot be excluded. However, their distribution and morphology suggest that they most likely originate from primary sensory neurons in the TG (Silverman and Kruger, 1989; Ichikawa et al., 2000; Ichikawa and Sugimoto, 1997).

Our immunohistochemical analysis of wildtype and knockout mice indicated that *Brn-3a* deficiency had effects on the expression of neurochemical substance in the TG. In knockout mice, the number of parvalbumin-IR neurons greatly decreased in the TG. Correspond-

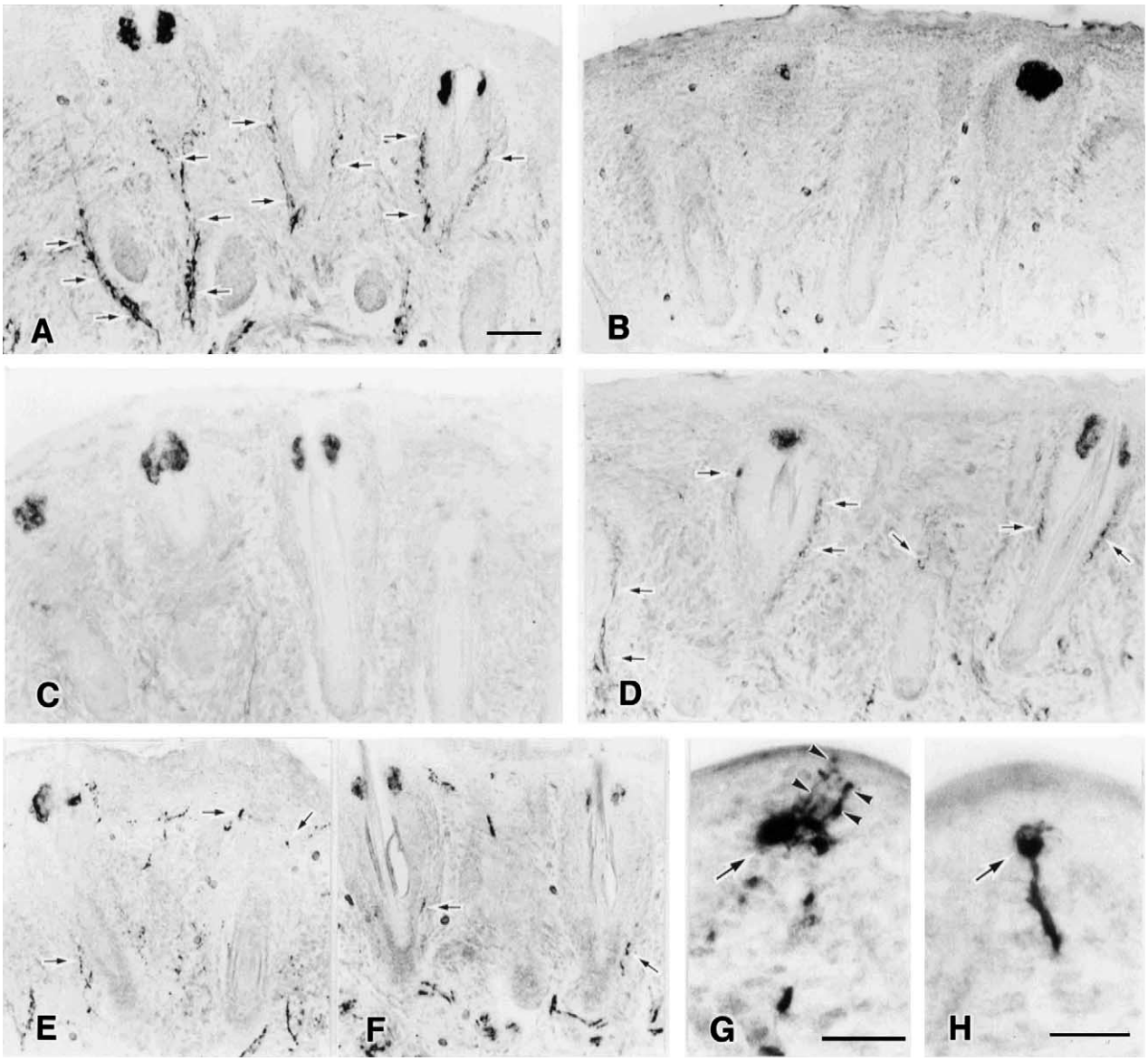

Fig. 8. Immunohistochemical microphotographs of parvalbumin- (A, B), calbindin D-28k- (C, D, G, H) and CGRP-IR (E, F) in the vibrissa (A-F) and palate (G, H) of *Brn-3a* wildtype (A, C, E, G) and knockout (B, D, F, H) mice at E18.5 (A-D, G, H) and P0 (E, F). The vibrissa is innervated by numerous parvalbumin-IR endings in the wildtype mouse (arrows in A) but not in the knockout mouse (B). In the wildtype mouse, the vibrissa is devoid of calbindin D-28k-IR innervation (C). In the knockout mouse, however, many calbindin D-28k-IR endings appear in the vibrissa (arrows in D). The distribution of CGRP-IR fibers and endings is similar in wildtype (arrows in E) and knockout (arrows in F) mice. Although the hard palate in both wildtype (an arrow in G) and knockout (an arrow in H) mice contains calbindin D-28k-IR corpuscular endings, the calbindin D-28k-IR intraepithelial endings are seen only in the wildtype mouse (arrowheads in G). Scale bars = 100 μm (A–F) and 50 μm (G, H).

ingly, the number of parvalbumin-IR fibers markedly decreased in the ION and parvalbumin-IR endings disappeared in the vibrissa. Therefore, the loss of *Brn-3a* probably causes a reduction of large and medium-sized parvalbumin-IR neurons which innervate the vibrissa through the ION.

Unlike the parvalbumin-IR neurons, the number of calretinin-IR TG cells greatly increased in *Brn-3a* knockout mice. The TG was almost devoid of calretinin-IR cells in wildtype mice. In knockout mice, however, many small and medium-sized cells showed calretinin-IR. In addition, calretinin-IR fibers appeared in the ION of the mutant. Therefore, the absence of *Brn-3a* probably increased the number of calretinin-IR neurons in the TG. Similar to calretinin-IR neurons, the number of calbindin D-28k-IR neurons dramatically increased in the knockout mice. Correspondingly, the number of calbindin D-28k-IR nerve fibers increased in the ION and MN, and numerous calbindin D-28k-IR endings appeared in the vibrissal follicle. By cell size analysis, the proportion of small calbindin D-28k-IR neurons increased but those of large immunoreactive neurons decreased in the mutant mouse. These findings indicate a significant increase in the number of small calbindin

D-28k-IR neurons in the mutant. It is unlikely that the increase in calretinin- and calbindin D-28k-IR neurons is due to differential apoptosis of TG cells that require Brn-3a for survival. However, it still remains unclear whether Brn-3a has an effect on the proliferation of these neurons or the expression of calretinin and calbindin D-28k. It may shed some light on this to investigate whether the expression is increased in *Brn-3a* knockout mouse before the apoptosis of neurons that require *Brn-3a* for survival.

No obvious change was observed for the number and peripheral distribution of CGRP-IR neurons in *Brn-3a* knockout mice. However, the cell size spectra of CGRP-IR TG neurons were quite different between wildtype and knockout mice. The proportion of medium-sized and large CGRP-IR neurons decreased while that of small CGRP-IR neurons substantially increased in the knockout mouse. These findings suggest that *Brn-3a* deficiency causes an increase in the number of small CGRP-IR neurons and a decrease in the number of larger CGRP-IR neurons. The lack of *Brn-3a* probably increases CGRP-IR expression in small TG neurons. However, 70% of TG neurons die by embryonic day 16.5 in *Brn-3a* knockout mice (Huang et al., 1999). A recent study demonstrated axonal growth defects and a failure to correctly innervate whisker follicles, all of which precede sensory neural death in the knockout mouse (Eng et al., 2001). The axon growth and survival of large CGRP-IR neurons may depend on *Brn-3a* in the TG.

CONCLUSION

The present study investigated the effect of *Brn-3a* deficiency on parvalbumin-, calbindin D-28k-, calretinin- and CGRP-IR TG neurons. The loss of *Brn-3a* reduced the number of medium-sized to large parvalbumin-IR neurons which supplied the vibrissal innervation. In contrast, the numbers of small calbindin D-28k- and calretinin-IR TG neurons which innervated the vibrissa and/or facial skin increased in the knockout mouse. The lack of *Brn-3a* may have different effects on small and large CGRP-IR TG neurons. These data together suggest that *Brn-3a* deficiency has effects on the expression of neurochemical substances in the TG.

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