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Short communication

Brn-3a deficiency increases tyrosine hydroxylase-immunoreactive neurons in the dorsal root ganglion

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Abstract

Immunohistochemistry for tyrosine hydroxylase (TH) was performed on the dorsal root ganglia (DRG) in wild-type, heterozygous and *Brn-3a* knockout mice at embryonic day 18.5. TH-immunoreactive (-IR) neurons were detected in the DRG of wild-type and heterozygous mice, but their proportion was greatly increased by the loss of *Brn-3a* function (wild-type and heterozygot, 8.4%; knockout, 20.9%). IR neurons were of various sizes in wild-type (mean ± S.D. = 118.1 ± 55.4 μm^2, range = 26.6–306.3 μm^2) and heterozygous mice. In the knockout mice, however, TH-IR neurons were mostly small (mean ± S.D. = 68.2 ± 34.3 μm^2, range = 11.8–166.8 μm^2).

The present study suggests that *Brn-3a* may normally suppress TH expression in many small DRG neurons but activate TH expression in large DRG neurons.

Theme: Sensory systems
Topic: Somatic and visceral afferents

Keywords: Brn-3a; Calcitonin gene-related peptide; Dorsal root ganglion; Immunohistochemistry; Knockout mouse; Tyrosine hydroxylase

Brn-3a/Brn-3.0 is a POU domain transcription factor which is predominantly expressed by neurons [3,5,6,15,19]. It can induce expression of the *Bcl-2* gene and protect neurons from apoptosis [2,15,16,19]. Brn-3a can also activate a number of other neuronally expressed genes and stimulate neuronal process outgrowth [15,17,18]. During mouse development, the trigeminal and dorsal root ganglia (DRG) show intense *Brn-3a* expression [20,21]. Previous studies have demonstrated that targeted deletion of the *Brn-3a* gene in mice results in a marked reduction of neurons in the trigeminal ganglion [6,21]. In the *Brn-3a* mutants, only 30% of the normal complement of neurons survive until birth [6]. However, the loss of DRG neurons is not apparent in the mutant mouse [21]. Recently, the effect of *Brn-3a* deficiency on the expression of calcitonin gene-related peptide (CGRP) has been reported in the mouse embryo [12]. CGRP-containing neurons are abundant in the DRG of both wild-type and mutant mice. However, the number of medium-sized CGRP-IR neurons decreases, and that of small IR neurons increases in the mutant DRG. This suggests that Brn-3a deficiency may affect the cell body size and neurochemical content of primary nociceptors in the DRG.

Tyrosine hydroxylase (TH) is a rate-limiting enzyme of catecholamine synthesis. This enzyme is localized to small- to medium-sized neurons in the cranial sensory ganglia

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[4,8,9,14]. In the glossopharyngeal sensory ganglion, TH-containing neurons innervate the carotid body and are considered to be chemoreceptors [4,9]. In the mouse DRG, however, TH-containing neurons appear only at the embryonic stage [13]. Such neurons cannot be detected in the adult DRG. Therefore, TH appears to be transiently expressed by DRG neurons during development. The physiological function has been unclear. However, TH expression may be associated with survival, axonal growth and/or terminal formation of DRG neurons at embryonic stage.

In this study, immunohistochemistry for TH was performed on the DRG of *Brn-3a* knockout mice to explore the possibility that Brn-3a may be involved in the transient expression of this enzyme. The coexpression of TH and CGRP was also investigated by a double immunofluorescence method.

Mice lacking the expression of *Brn-3a* gene were prepared as described by Xiang et al. [21]. Two wild-type, four heterozygous and four homozygous mutant mice at embryonic day 18.5 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 vertebral column, including the DRGs and spinal cords at the thoracic and lumbar levels, was dissected, immersed in PBS containing 20% sucrose overnight, frozen-sectioned transversely at 10 μm and thaw-mounted on gelatin-coated glass slides.

For demonstration of TH-IR, sections were incubated with rabbit anti-TH serum (1:50,000; Chemicon, USA) followed by the incubation with biotinylated goat antirabbit IgG and avidin–biotin–horseradish peroxidase complex

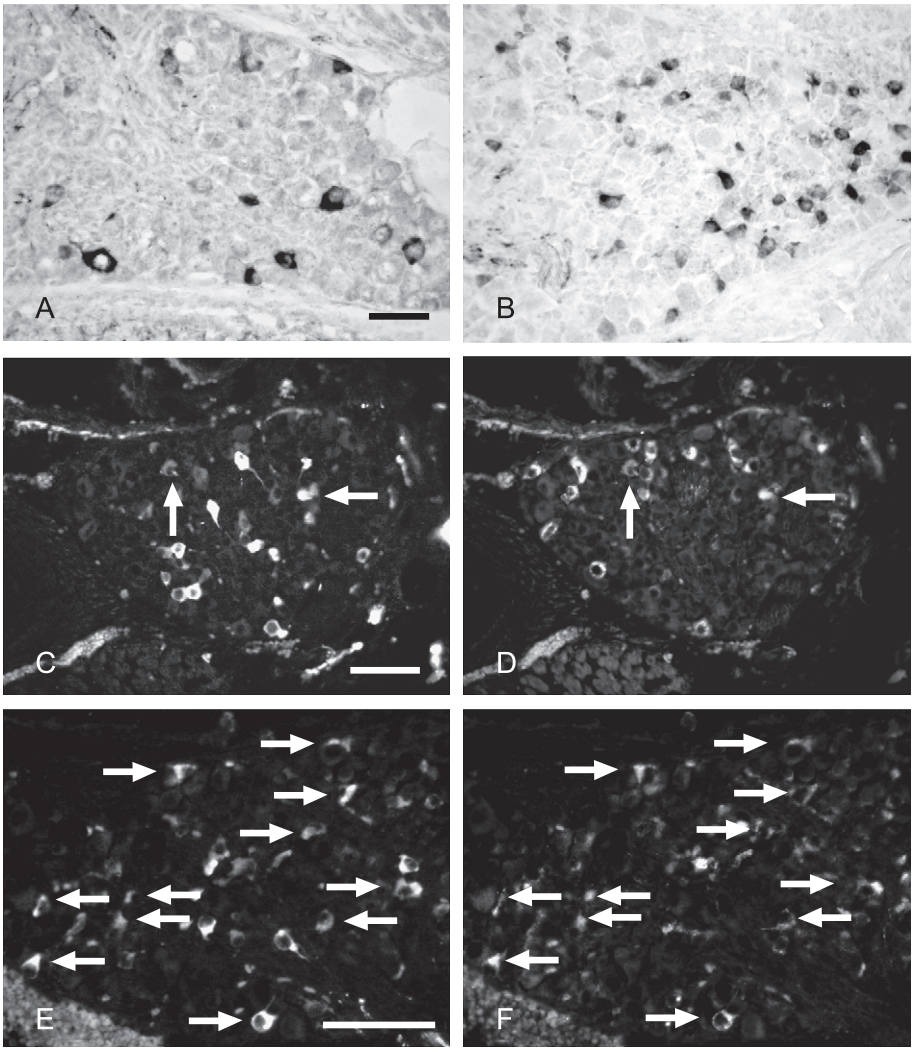

Fig. 1. Immunohistochemical microphotographs of TH-IR (A–C, E) and CGRP-IR (D, F) in the DRG of wild-type (C, D), heterozygous (A) and *Brn-3a* knockout (B, E, F) mice at E18.5. The number of TH-IR neurons increases in *Brn-3a* knockout mice (B) compared to wild-type and heterozygous mice (A). TH-IR neurons are of various sizes in wild-type and heterozygous mice (A), whereas such neurons are mostly small in knockout mice (B). Panels (C) and (D), and (E) and (F) are from the same fields of view, respectively. Double immunofluorescence reveals the coexpression of TH and CGRP in wild-type and knockout mice. DRG neurons which coexpress TH and CGRP are abundant in the knockout mouse (E, F), whereas such neurons are relatively rare in the wild-type mouse (C, D). Bars = 50 (A) and 100 μm (C, E). Panels (A) and (B), (C) and (D) and (E) and (F) are at the same magnifications, respectively.

(Vector Laboratories). The microscopic image (×215) of TH-IR and -negative cell bodies was projected over a digitizer tablet using a drawing tube. Outline of their cytoplasm and presence of the nucleolus were examined with light or polanret microscopy. The cross-sectional area of those cell bodies that contained the nucleolus was recorded. The data were stored into a computer (NEC, Japan) and analyzed. In this study, all TH-IR cells were considered to be neurons because they had a round appearance and were larger than glial cells (diameter > 4 μm).

To colocalize TH and CGRP in the DRG, sections were incubated with a mixture of rabbit anti-TH serum (1:1000) and guinea-pig anti-CGRP serum (1:1000, Milab, Sweden) overnight at room temperature. Subsequently, sections were incubated in a mixture of lissamine rhodamine B chloride-conjugated donkey antirabbit IgG (1:500, Jackson ImmunoResearch Labs, USA) and fluorescein isothiocyanate-conjugated donkey anti-guinea-pig IgG (1:100, Jackson ImmunoResearch Labs).

The specificity of the primary antiserum has been described elsewhere [7,8].

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.

The DRG contained many TH-IR neurons in wild-type and heterozygous mice (Fig. 1A). Such neurons were numerous in the thoratic DRGs compared to the lumbar DRGs. The IR was detected in the cytoplasm but not in the nucleus of these neurons. TH-IR nerve fibers were also observed within the DRG. The distribution of TH-IR neurons and nerve fibers was similar in wild-type and heterozygous mice. However, the loss of *Brn-3a* significantly increased the number of TH-IR neurons in the thoratic and lumbar DRGs (Fig. 1B). The mean proportions and S.E. per section of TH-IR neurons in wild-type and heterozygous mice, and knockout mice were 8.4 ± 1.4% and 20.9 ± 3.8%, respectively. The data were obtained from 2769 neurons in four sections of two wild-type mice, 1079 neurons in four sections of two heterozygous mice and 1128 neurons in eight sections of four knockout mice. The difference between wild-type and heterozygous mice, and knockout mice was statistically significant ($p < 1\%$, ANOVA). By contrast, the distribution of TH-IR nerve fibers was similar in the DRG of wild-type, heterozygous and knockout mice; some TH-IR nerve fibers were seen running across the ganglion.

Cell size analysis showed that TH-IR neurons were of various sizes in wild-type (mean ± S.D. = 118.1 ± 55.4 μm^2, range = 26.6–306.3 μm^2, n = 113; Fig. 2) and heterozygous mice. In wild-type mice, 40% (45/113) of TH-IR neurons was smaller than 100 μm^2 and a half (51.3% or 58/113) measured from 100 to 200 μm^2. About 10% (10/113) of TH-IR neurons had cell bodies > 200 μm^2. In knockout mice, the proportion of small TH-IR neurons greatly increased (Fig. 2, mean ± S.D. = 68.2 ± 34.3 μm^2, range = 11.8 to 166.8 μm^2, n = 257). Among these, 81.7% (210/257) of TH-IR neurons were smaller than 100 μm^2. Only 18.3% (47/257) measured from 100 to 200 μm^2. TH-IR neurons > 200 μm^2 could not be detected in the mutant mice.

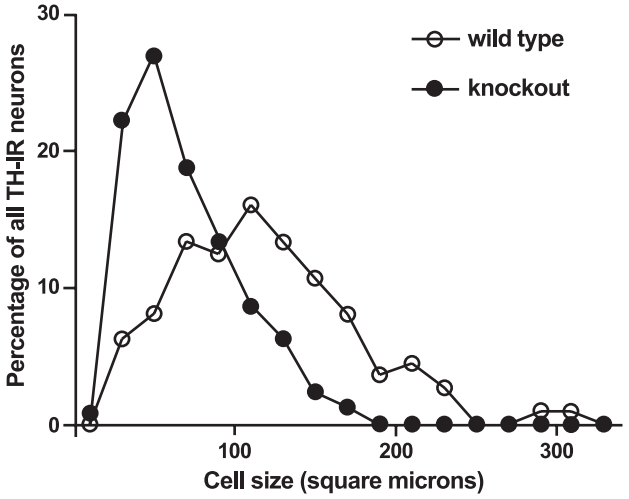

Fig. 2. Cell size spectra of TH-IR DRG neurons in wild-type and *Brn-3a* knockout mice. The data were obtained from 113 and 257 TH-IR neurons in wild-type and knockout mice, respectively.

Double immunofluorescence revealed coexpression of TH and CGRP in the DRG. In wild-type and heterozygous mice, the coexpression was rare (Fig. 1C,D). In the knockout mouse, however, the number of DRG neurons which coexpressed these substances dramatically increased (Fig. 1E,F). The mean numbers ± S.E. per section of such neurons in wild-type and knockout mice were 1.0 ± 0.2 (n = 20) and 10.6 ± 1.4 (n = 17), respectively. The difference between these mice was statistically significant ($p < 1\%$, t-test). In the wild-type mouse, 15.1% (19/126) of TH-IR neurons was also immunoreactive for CGRP. Only 3.3% (19/569) of CGRP-IR neurons coexpressed TH-IR. In the knockout mice, however, 56.1% (180/321) of TH-IR neurons showed CGRP-IR, and 30.2% (180/597) of CGRP-IR neurons contained TH-IR.

The present study analyzed the distribution of TH-IR in the DRG of wild-type, heterozygous and *Brn-3a* knockout mice. In the knockout mice, we found that the number of TH-IR neurons greatly increased. In a recent microarray study, TH expression was down-regulated in the TG of *Brn-3a* knockout mice [1]. Therefore, there appear to be different effects of Brn-3a deficiency on TH expression in the DRG and TG. Our cell size analysis further revealed a significant difference in the size spectrum of TH-IR DRG neurons between wild-type and knockout mice. TH-IR neurons were of various sizes in the wild-type mouse. In the knockout mouse, however, the proportion of small TH-IR neurons greatly increased. These findings indicate that the absence of *Brn-3a* increased the number of small TH-IR

neurons in the DRG. In addition, our double immunofluorescence analysis revealed the coexpression of TH and CGRP. Compared to wild-type mice, DRG neurons coexpressing these substances greatly increased in the mutant. Thus, the expression of both TH and CGRP may be elevated in small DRG neurons by *Brn-3a* deficiency.

In the present study, we found that large TH-IR neurons, which were present in wild-type mice, disappeared in *Brn-3a* knockout mice. This is similar to our previous finding that the number of large CGRP-IR neurons decreased in the mutant TG and DRG [11,12]. However, the mechanism of their reduction appears to be different between these two ganglia. Because most large neurons are lost in the mutant TG [10], the survival of large CGRP-IR neurons is considered to depend on *Brn-3a* in the TG. However, the extra cell death has never been reported in the mutant DRG. Therefore, it is likely that TH and CGRP expression in large DRG neurons may be suppressed by *Brn-3a* disruption and that *Brn-3a* may be able to up-regulate their expression in large DRG neurons. If this is the case, Brn-3a may have different functions in regulating the expression of neurochemical substances in small and large DRG neurons.

In conclusion, our present study investigated the effect of *Brn-3a* deficiency on the number and size of TH-IR DRG neurons. The loss of *Brn-3a* causes a significant increase of small TH-IR neurons and eliminates large TH-IR neurons. In addition, it dramatically increases the number of DRG neurons which coexpress TH and CGRP. These data suggest that *Brn-3a* disruption may elevate TH and CGRP expression in small DRG but suppress TH expression in large DRG neurons.

Acknowledgments

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