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Short Communication

Brn-3a is required for the generation of proprioceptors in the mesencephalic trigeminal tract nucleus

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Abstract

The distribution of motor and proprioceptive neurons was investigated in the trigeminal nervous system of wild-type and *Brn-3a* knockout mice at embryonic day 18.5 and postnatal day 0. We found that the trigeminal motor nucleus (Mo5) contained abundant motoneurons in wild-type (mean number ± SD per section = 128 ± 22, range = 93–167) and knockout (mean number ± SD per section = 121 ± 23, range = 75–158) mice and that the cell size of Mo5 neurons was similar between these mice (wild-type, mean ± SD = 165 ± 59 μm^2, range = 65–326 μm^2; knockout, mean ± SD = 167 ± 59 μm^2, range = 71–327 μm^2). Mo5 neurons were immunoreactive for calcitonin gene-related peptide and such immunoreactive neurons were abundant in both wild-type and mutant mice. In the mesencephalic tract nucleus (Mes5) of wild-type mice, many proprioceptors (mean number ± SD per section = 56 ± 19, range = 27–85) that contained parvalbumin immunoreactivity were also observed. In knockout mice, however, Mes5 neurons could not be detected. The area of brainstems which normally contained the Mes5 was devoid of parvalbumin-immunoreactive proprioceptors. The present study suggests that *Brn-3a* is required for the development of proprioceptors but not motoneurons in the trigeminal nervous system.

Theme: Motor systems and sensorimotor integration
Topic: Spinal cord and brainstem

Keywords: Transcription factor; Knockout mouse; Parvalbumin; Proprioceptor; Immunohistochemistry

Brn-3a is a member of the POU family of transcription factors which is predominantly expressed by neurons [5,6,7,18,24]. This factor can induce expression of the *Bcl-2* gene and is thought to protect neurons from apoptosis [3,15,18,23]. In addition, Brn-3a can activate a number of other neuronally expressed genes and stimulate neuronal process outgrowth [15,21,22]. During the mouse development, the trigeminal ganglion shows intense *Brn-3a* expression [24]. Targeted deletion of the *Brn-3a* gene results in a marked reduction of neurons in the trigeminal ganglion [7,18,25]. Therefore, the survival of exteroceptive primary sensory neurons is probably dependent upon Brn-3a in the trigeminal ganglion. In our previous studies, we have shown that low-threshold mechanoreceptors and nociceptors in orofacial regions are sensitive to the loss of *Brn-3a* [11,12]. In addition, the knockout mouse disrupts the rhythmic jaw opening and closing movement [25]. This may suggest that the deficiency has some effect on motor and/or proprioceptive neurons in the trigeminal nervous system.

Cell bodies of trigeminal motoneurons and proprioceptors are located in the trigeminal motor (Mo5) and mesencephalic trigeminal tract (Mes5) nuclei, respectively. These neurons innervate masticatory muscles and play an important role in jaw movement. Periodontal ligaments also receive proprioceptive innervation from the Mes5. Previous

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immunohistochemical studies have demonstrated that motoneurons express calcitonin gene-related peptide (CGRP) [1]. In the musculature, motor end plates are immunoreactive for the peptide [19,20]. On the other hand, primary proprioceptors express parvalbumin, a member of calcium-binding protein family [10,16,17]. In the Mes5, parvalbumin-containing neurons send their peripheral axons to muscle spindles [17]. In addition, Brn-3a expression has been shown in Mes5 neurons during development [8].

In this study, the distribution of Mo5 and Mes5 neurons was examined in wild-type and *Brn-3a* knockout mice to investigate the effect of *Brn-3a* deficiency on motor and proprioceptive neurons in the trigeminal nervous system.

Mice lacking expression of *Brn-3a* were prepared according to the method of Xiang et al. [25]. Four wild-type and 4 knockout mice at embryonic day 18.5 (E18.5) and 1 wild and 1 knockout mice at postnatal day 0 (P0) were obtained from mating of the heterozygous mice. Animals were killed by decapitation and immersed overnight in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C. Subsequently, tissues were stored at 4 °C in phosphate-buffered saline (PBS) containing 0.1 mM sodium azide until used. The brainstem and masseters were dissected, immersed in PBS containing 20% sucrose overnight. The materials were frozen sectioned at 10 μm for the masseter or 30 μm for the brainstem and thaw-mounted on gelatin-coated glass slides. The sections of brainstems were processed for Nissl stain. Some sections were also stained for CGRP or parvalbumin immunoreactivity (IR). They were incubated with rabbit anti-CGRP serum (1:50,000, Peninsula, USA) or rabbit anti-parvalbumin serum (1:50,000, Swant, Switzerland) followed by the incubation with biotinylated goat anti-rabbit IgG and avidin–biotin–horseradish peroxidase complex (Vector Laboratories). Immunoreaction products were visualized with diaminobenzidine and nickel ammonium sulfate. For cell size analysis of Nissl-stained neurons, microscopic images (×215) of cell bodies were projected over a digitizer tablet using a drawing tube. The cross-sectional area of those cell bodies that contained the nucleolus was recorded. The statistical analysis was conducted in wild-type and *Brn-3a* knockout mice at E18.5 because the distribution of Mes5 neurons was similar at P0 and E18.5.

The specificity of the primary antisera used in this study has been described elsewhere [9,11].

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 Mo5 contained numerous neurons in wild-type and *Brn-3a* knockout mice (Figs. 1A and B). The mean number ± SD per section of such neurons in the knockout mice (121 ± 23, range = 75–158) was similar to that for wild-type mice

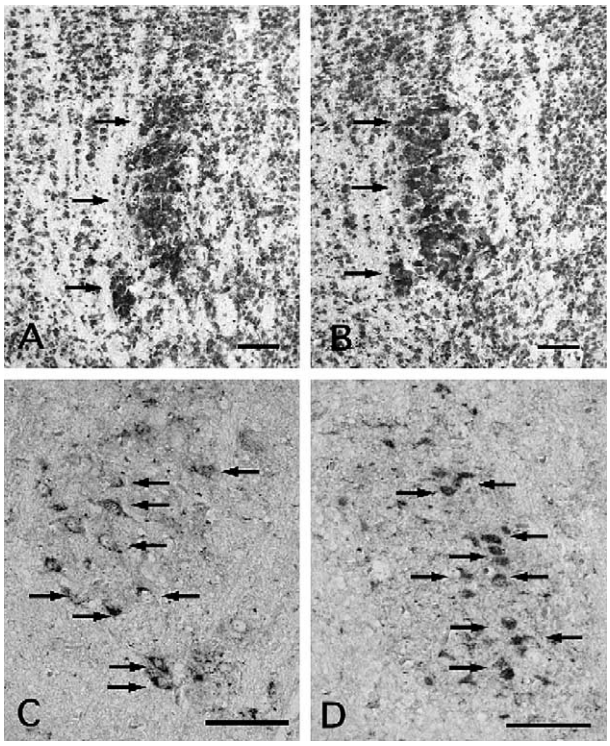

Fig. 1. Microphotographs of Nissl stain (A, B) and CGRP-IR (C, D) in the Mo5 of wild-type (A, C) and *Brn-3a* knockout (B, D) mice at E18.5 (A, B) and P0 (C, D). The Mo5 contained many motoneurons in wild-type (arrows in panel A) and *Brn-3a* knockout (arrows in panel B) mice. The distribution of CGRP-IR Mo5 neurons was similar in these mice (arrows in panels C, D). Scale bars = 100 μm.

(128 ± 22, range = 93–167, $P > 0.25$, Mann–Whitney). The data were obtained from 8 sections of 4 wild-type mice and 9 sections of 4 *Brn-3a* knockout mice at E18.5. In addition, the cell size spectrum of Mo5 neurons was similar between the wild-type (mean ± SD = 165 ± 59 μm^2, range = 65–326 μm^2, n = 101) and *Brn-3a* knockout (mean ± SD = 167 ± 59 μm^2, range = 71–327 μm^2, n = 100) mice (Fig. 2). More than half of Mo5 neurons fell in the range of 100–200 μm^2 (wild-type, 64% or 65/101; *Brn-3a* knockout, 62% or 62/100). Twenty-five percent of Mo5 neurons were larger than 100 μm^2 (wild-type, 24/101; *Brn-3a* knockout, 25/100). Mo5 neurons < 100 μm^2 were relatively rare in these mice (wild-type, 12% or 12/101; *Brn-3a* knockout, 13% or 13/100). Our immunohistochemical analysis also revealed that the Mo5 neurons contained abundant CGRP-immunoreactive (IR) neurons in both wild-type and *Brn-3a* knockout mice (Figs. 1C and D). In these CGRP-IR neurons, IR granules were distributed throughout the cytoplasm.

Many Mes5 neurons were detected in wild-type mice (Fig. 3A). The mean number ± SD per section of such neurons was 56 ± 19 (range = 27–85). The data were obtained from 14 sections of 4 wild-type mice at E18.5. The cell size analysis demonstrated that Mes5 neurons were smaller than Mo5 neurons (mean ± SD = 88 ± 47 μm^2, range = 20–283 μm^2, n = 102). More than half (65% or 66/102) of Mes5 neurons were smaller than 100 μm^2. About

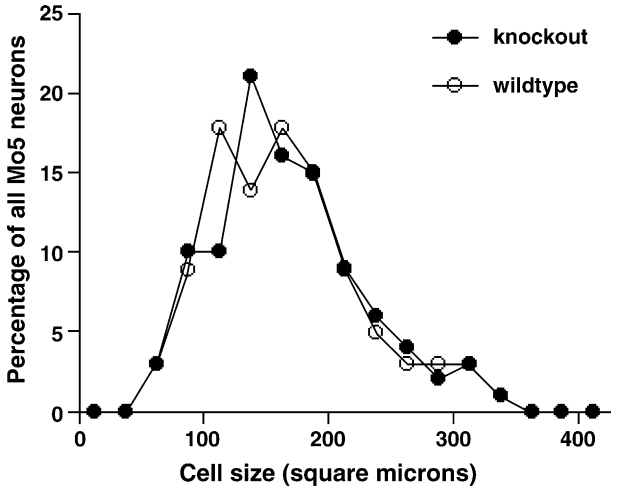

Fig. 2. Cell size spectra of Mo5 neurons in wild-type and *Brn-3a* knockout mice at E18.5. The data were obtained from 101 and 100 neurons in wild-type and *Brn-3a* knockout mice, respectively.

30% (33/102) fell in the range of 100–200 μm^2. Mes5 neurons >200 μm^2 were very rare (3% or 3/102). In the Mes5, proprioceptive neurons contained parvalbumin-IR (Fig. 3C). The IR was observed in the cytoplasm but not in the nucleus of these neurons. However, Mes5 neurons disappeared in the knockout mice (Fig. 3B). Parvalbumin-IR proprioceptors could not be detected in the area of brainstems which normally contained the Mes5 (Fig. 3D).

In the masseter of wild-type mice, CGRP-IR nerve fibers were located around blood vessels (figure not shown). IR fibers were also detected in the vicinity of muscle fibers. The distribution of CGRP-IR fibers was similar in wild-type and *Brn-3a* knockout mice. By contrast, parvalbumin-IR nerve fiber was detected in the muscle of neither wild-type nor *Brn-3a* knockout mice.

The present study investigated the distribution of Mo5 and Mes5 neurons in wild-type and *Brn-3a* knockout mice. In these mice, the number and cell size of Mo5 neurons were barely affected by the deficiency. In the Mes5, however, the loss of *Brn-3a* caused disappearance of primary sensory neurons, indicating that the survival of Mes5 neurons depends on *Brn-3a*. In a previous study, *Brn-3a* knockout mice were shown to be defective of suckling and have no milk in their stomachs [25]. They displayed no rhythmic opening and closing jaw movements in response to tactile stimulation of lips. Such a dysfunction is probably associated with the absence of Mes5 neurons because Mes5 neurons include muscular proprioceptors in the trigeminal nervous system. It is unclear whether or not Mes5 neurons are generated initially in *Brn-3a* knockout mice. In addition, the possibility that Mes5 neurons may generate normally but migrate aberrantly to other regions can not be excluded in this study. However, the loss of *Brn-3a* may deprive proprioception and coordinated movements of masticatory muscles.

Brn-3a knockout mice have been shown to exhibit uncoordinated limb movement [25], suggesting that Brn-3a deficiency may affect muscular proprioceptors in the spinal nervous system. In this system, muscular proprioceptors are located in the dorsal root ganglion. These neurons have large cell bodies and contain parvalbumin-IR [2]. Our previous study has demonstrated that the number of parvalbumin-IR neurons in the dorsal root ganglion and the distribution of their peripheral axons in muscle spindles are similar in wild-type and *Brn-3a* knockout mice [13]. However, the number of parvalbumin-IR fibers and terminals is decreased in the spinal cord of the knockout mice. Therefore, *Brn-3a* is thought to be involved in the central projection and terminal formation of proprioceptors in the spinal cord. Unlike in the dorsal root ganglion, trigeminal proprioceptors are lost in the knockout mouse. Thus, it is likely that *Brn-3a* has different functions in spinal and trigeminal proprioceptors.

Brn-3a is thought to control the survival and differentiation of trigeminal neurons by regulating the expression of neurotrophin receptors. In the trigeminal ganglion of *Brn-3a* knockout mice, the surviving neurons express glial cell-derived neurotrophic factor receptor but not Trk receptors [7]. Previous studies have demonstrated that the spinal and trigeminal proprioceptors also have different neurotrophin dependency. In the dorsal root ganglion,

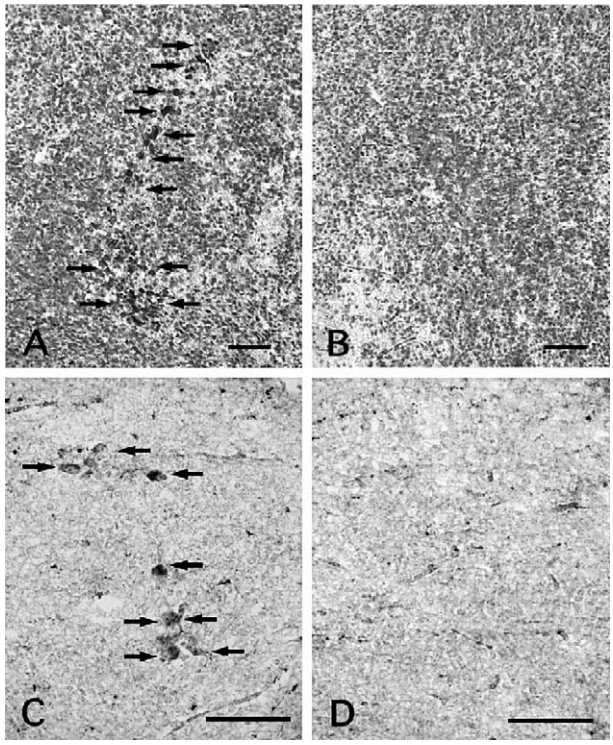

Fig. 3. Microphotographs of Nissl stain (A, B) and parvalbumin-IR (C, D) in the Mes5 region of wild-type (A, C) and *Brn-3a* knockout (B, D) mice at E18.5 (A, B) and P0 (C, D). Many Mes5 neurons are observed in wild-type mice (arrows in panel A) but not in *Brn-3a* knockout mice (B). In wild-type mice, Mes5 neurons are immunoreactive for parvalbumin (arrows in panel C). However, the area of brainstems which normally contain the Mes5 is devoid of parvalbumin-IR neurons in the knockout mice (D). Scale bars = 100 μm.

proprioceptors express TrkC and their survival is dependent upon neurotrophin-3 [14]. However, the development of Mes5 neurons requires both brain-derived neurotrophic factor and neurotrophin-3 that act through TrkB and TrkC [4,17]. At present, the relationship between *Brn-3a* and neurotrophin dependency in Mes5 neurons remains unclear. Further studies will be necessary to know whether *Brn-3a* in trigeminal proprioceptors is involved in the regulation of neurotrophin receptor expression.

In conclusion, the present study has examined Mo5 and Mes5 neurons in *Brn-3a* knockout mice. Loss of *Brn-3a* function does not appear to have any effect on the development of motoneurons in the trigeminal nervous system. However, it causes a complete loss of Mes5 neurons, indicating that *Brn-3a* is involved in the generation of trigeminal proprioceptors.

Acknowledgments

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