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Development of the mesencephalic trigeminal nucleus requires a paired homeodomain transcription factor, *Drg11*

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Abstract

The mesencephalic trigeminal nucleus (Me5) innervates muscle spindles and is responsible for receiving and transmitting proprioception from the oro-facial region. Molecular mechanisms underlying the development of the Me5 are poorly understood. Evidence is provided here that transcription factor *Drg11* is required for Me5 development. *Drg11* was expressed in the Me5 cells of the embryonic and early postnatal mouse brains, and the Me5 cells were absent in *Drg11*$^{-/-}$ mice at birth. The absence of the Me5 cells in *Drg11*$^{-/-}$ mice appeared to be caused by increased cell death in the Me5 during embryonic development. In postnatal *Drg11*$^{-/-}$ mice, Me5 cell innervation of masseter muscle spindles was undetectable, while robust trigeminal motoneuron innervation of masseter muscle fibers was detected. The postnatal bodyweight of *Drg11*$^{-/-}$ mice was notably less than that of wild-type mice, and this might result, in part, from disruption of the oro-facial proprioceptive afferent pathway. Taken together, our results demonstrate an essential role for *Drg11* in the development of the Me5.

Keywords

Drg11; Transcription factor; Mesencephalic trigeminal nucleus; Development; Cell survival

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Introduction

The mesencephalic trigeminal nucleus (Me5) contains the primary sensory neurons that innervate the muscle spindle of the masticatory and other muscles in the oro-facial region, as well as a small number of periodontal mechanoreceptive afferents. The Me5 neurons convey proprioceptive sensory information from oro-facial muscles to the trigeminal motor nucleus (Mo5) and other motor nuclei in the brainstem (e.g. nucleus of the facial nerve) via the monosynaptic stretch reflex circuit for the coordination of muscle contraction and, therefore, are involved in masticatory and suckling behaviors (Usunoff et al., 1997; Marani and Usunoff, 1998; Lazarov, 2000).

Unlike other primary sensory neurons whose cell bodies are located in peripheral ganglia, Me5 contains the only primary sensory neurons located within the central nervous system (Capra and Wax, 1989; Luo and Li, 1991; Luo et al., 1991; Porter and Donaldson, 1991). These cells are believed to be derived from the mesencephalic neural crest (Narayanan and Narayanan, 1978; Stainier et al., 1991; Stainier and Gilbert, 1991). Me5 neurons are the first-born neurons in the mesencephalon, and their axons pioneer some of the major nerve tracts in the brain. However, the molecular mechanism underlying the development of the Me5 is poorly understood. *Brn3a*, a member of the POU family of transcription factors, is expressed in the Me5, and Me5 neurons are lost in *Brn3a*$^{-/-}$ mice at postnatal day 0 (P0) (Xiang et al., 1996; Ichikawa et al., 2005). *Krox-20*, a C2H2-type zinc-finger transcription factor, impacts the final number of Me5 neurons by regulating the period and extent of apoptosis in the Me5 during development (De et al., 2005). In addition, the number of Me5 neurons is significantly reduced in trkC$^{-/-}$ mice, indicating that NT-3/trkC signaling is required for the survival of most Me5 neurons (Matsuo et al., 2000).

Drg11, a paired homeodomain transcription factor, was initially cloned from a differential hybridization aimed at identifying dorsal root ganglia (DRG)-specific genes (Saito et al., 1995). *Drg11* is required for nociceptive circuitry assembly in the spinal dorsal horn and the survival of nociceptive neurons in DRG (Chen et al., 2001; Rebelo et al., 2006). *Drg11* is also important for the formation of the whisker-related somatosensory maps in the principal sensory nucleus of the trigeminal nerve, thalamus and somatosensory cortex (Ding et al., 2003). In the present study, we set out to ascertain the role of *Drg11* in the development of the Me5.

Results

Expression of Drg11 in the Me5

The spatiotemporal expression patterns of *Drg11* in the Me5 were examined by *in situ* hybridization. *Drg11* expression was first detected in the presumptive Me5 at E10.5 (data not shown). At E11.5, *Drg11*-expressing cells in the Me5 were aligned along the lateral wall of the mesencephalon and metencephalon. As shown in Fig. 1A,*Drg11* expression was restricted to the Me5 in the mesencephalon, while at the level of the rostral metencephalon it was also observed in the principal sensory nucleus of the trigeminal nerve and trigeminal ganglia, but not in the motor nucleus of the trigeminal nerve, consistent with a previous report (Ding et al., 2003). A similar*Drg11* expression pattern was observed at E13.5, except

that there appeared to be a greater number of *Drg11*-expressing cells in the Me5 (Fig. 1B). From E15.5 to E17.5, *Drg11*-expressing neurons appeared to be less numerous in the Me5 (Figs. 1C, D), possibly reflecting excessive apoptosis there (Alley, 1974). At early postnatal stages, *Drg11* expression was found in the Me5 at P7 (Fig. 1E), but *in situ* signals became very weak at P14 and disappeared around P21 (data not shown). Thus, *Drg11* is robustly expressed in the developing Me5 and it may function in Me5 development.

Retrograde labeling of the developing Me5

Normal development of the Me5 was studied by DiI retrograde labeling. DiI crystals were inserted into the first branchial arch at E10.5-E11.5 or, at later ages, into the jaw-closing muscles, the peripheral target of most Me5 neurons. DiI-labeled cells were first observed in the presumptive Me5 at E11.5 and they were located along the lateral wall of the metencephalon (Figs. 2A, B). At E13.5, DiI-labeled neurons were observed in the Me5 in both the mesencephalon and metencephalon, and they were numerous (Figs. 2C, D), indicating an increase in the peripheral projections of Me5 neurons. Thereafter, the rostral Me5 in the mesencephalon and caudal Me5 in the metencephalon could be clearly identified. At E17.5, DiI-labeled Me5 neurons were located in the lateral periaqueductal gray of the midbrain and in the dorsolateral tegmentum of the pons (Figs. 2E, F), and a similar distribution pattern of DiI-labeled neurons in the Me5 was observed at P0 (Figs. 2G, H). Postnatally, Me5 neurons were parvalbumin-positive (Figs. 2I, J) (Lazarov, 2002).

Me5 integrity in Drg11$^{-/-}$ mice

To elucidate the role of *Drg11* in the Me5 development, we studied the integrity of the Me5 in *Drg11*$^{-/-}$ mice at P0 and P14. In Nissl-stained sections, the Me5 was clearly observed in the midbrain and pons of wild-type mice. Its cells are larger than those in the surrounding neuropil and stain very darkly for Nissl, thus allowing for their unambiguous identification. However, in *Drg11*$^{-/-}$ mice, no such cells were observed. To corroborate this observation, parvalbumin and cytochrome oxidase (CO) stainings were performed in other cases. These robust markers of Me5 cells also failed to reveal their presence in the brainstem of *Drg11*$^{-/-}$ mice (Figs. 3B, D and data not shown).

The absence of parvalbumin- and CO-positive Me5 cells in *Drg11*$^{-/-}$ mice might be due to a failure of synthesis of parvalbumin and loss of cytochrome oxidase enzyme activity in the absence of *Drg11* expression, thus leading to a false negative observation. To address this potential confounding, cholera toxin B subunit (CTB), a retrograde tracer, was applied to jaw-closing muscles to reveal Me5 neurons. In wild-type mice, CTB-labeled neurons were found in the Me5 of the midbrain and pons (Figs. 3E, G), but no CTB-labeled neurons were detected in the corresponding regions of *Drg11*$^{-/-}$ mice (Figs. 3F, H). Taken together, we conclude that the Me5 disintegrates in *Drg11*$^{-/-}$ mice during prenatal development.

Increased Me5 cell death in Drg11$^{-/-}$ embryos

All of the above suggest that *Drg11* is required for the survival of Me5 neurons during embryonic development. A variety of tools were employed to ascertain when and how Me5 cells die in *Drg11*$^{-/-}$ mice. At E11.5, Me5 neurons were retrogradely labeled with DiI in seemingly normal numbers and patterns in *Drg11*$^{-/-}$ embryos, as compared to wild-type

controls (data not shown). At 12.5, a normal increase in the prevalence of DiI-labeled Me5 neurons was also found in *Drg11*$^{-/-}$ embryos (Figs. 4A, B). This suggests that the initial generation, differentiation and migration of Me5 neurons and their peripheral projections are normal in *Drg11*$^{-/-}$ embryos. However, at E13.5, the number of DiI-labeled Me5 neurons decreased dramatically, relative to wild-type controls (approximately 266 ± 79 vs. 770 ± 46, $P < 0.01$) (Figs. 4C-F). The rostral Me5 was more severely affected in the mutants at this age, with near complete loss of DiI-labeled neurons at the midbrain level (Figs. 4C, D). In order to confirm this, neomycin phosphotransferase II (Neo) expression in the Me5 was analyzed in *Drg11*$^{+/-}$ and *Drg11*$^{-/-}$ embryos. In the generation of *Drg11*$^{-/-}$ mice, Neo was fused to the third coding exon of the *Drg11* locus, and thus Neo expression can be used to trace *Drg11*$^{+/-}$ and *Drg11*$^{-/-}$ cells (Chen et al., 2001; Ding et al., 2003). Neo-positive neurons were found in the Me5 of *Drg11*$^{+/-}$ embryos, but their number was dramatically reduced in *Drg11*$^{-/-}$ embryos at E13.5 (data not shown). At later stages (E15.5, E17.5 and P0), DiI-labeled and Neo-positive Me5 neurons were absent first in the rostral Me5 at E15.5, and then in the caudal Me5 at P0.

To determine whether the loss of the Me5 in *Drg11*$^{-/-}$ mice is due to abnormally excessive cell death in the Me5, caspase-3, a marker for apoptotic cells (Kothakota et al., 1997), was used. Caspase-3-positive cells were grossly more prevalent in the presumptive Me5 of *Drg11*$^{-/-}$ embryos at E12.5, as compared to wild-type embryos (approximately 39 ± 14 vs. 12 ± 1, $P < 0.01$) (Figs. 4G, H). In addition, TUNEL staining also revealed about twice as many profiles of dying cells in the lateral wall of the mesencephalon and metencephalon (approximately 106 ± 21 vs. 54 ± 3, $P < 0.01$; data not shown). Taken together, these data suggest that the initial generation of the Me5 is normal in *Drg11*$^{-/-}$ embryo, but that the survival of these neurons is dependent on the expression of *Drg11*.

Development of the Mo5 in Drg11$^{-/-}$ mice

Proprioceptive inputs from the oro-facial muscles are conveyed by the peripheral axons of Me5 cells. Their central axons project to the trigeminal motoneurons to form the masticatory monosynaptic stretch reflex circuitry which is necessary for normal masticatory behaviors. The absence of the Me5 in *Drg11*$^{-/-}$ mice must result in a significant loss of inputs to the trigeminal motor nucleus which might impact upon the integrity of the latter. To assess whether the gross morphology of the Mo5 is affected by the absence of *Drg11* expression, we first examined the developing Mo5 in *Drg11*$^{-/-}$ mice by DiI labeling. At embryonic stages E11.5-17.5, DiI-labeled trigeminal motor neurons in *Drg11*$^{-/-}$ embryos were indistinguishable from control embryos based upon gross inspection (data not shown). In wild-type mice, calcitonin gene-related peptide (CGRP)-positive neurons were detected in the Mo5 at P16 and similar expression of CGRP was observed in the Mo5 of *Drg11*$^{-/-}$ mice (Figs. 5A, B). Moreover, in the CTB tracing experiment as mentioned above, injection of CTB into jaw-closing muscles could also retrogradely label trigeminal motor neurons in the Mo5. It should be noted that CTB labeling in the Mo5 of *Drg11*$^{-/-}$ mice was similar to that of wild-type mice at P16 (Figs. 5C, D), indicating that the peripheral projections of trigeminal motor neurons are intact in the absence of *Drg11* expression.

To further assess innervation of masticatory muscles by trigeminal afferent and efferent fibers in *Drg11*$^{-/-}$ mice, DiI crystals were inserted into the trigeminal mandibular nerve. Motor terminals end on muscle fibers with elaborate motor endplates (Fig. 5E), whereas primary sensory endings terminate in the equatorial region of intrafusal muscle fiber with spiral- and ring-like shape (Fig. 5G). Similar to wild-type mice, motor nerve branches and their elaborate endings were observed in *Drg11*$^{-/-}$ mice (Figs. 5E, F). On the other hand, no sensory branches or endings or muscle spindles were observed in the mutant mice (Fig. 5H). An intact efferent innervation of masticatory muscles by the Mo5 in *Drg11*$^{-/-}$ mice was revealed by α-bungarotoxin labeling (Figs. 5I, J), which reveals postsynaptic acetylcholine receptors in muscle (Balice-Gordon and Lichtman, 1993). Taken together, our results show that the efferent innervation of the oro-facial muscles is intact, but that their afferent proprioceptive pathway is absent in *Drg11*$^{-/-}$ mice.

Postnatal survival, body weight regulation and feeding behavior of Drg11$^{-/-}$ mice

Whereas *Drg11* heterozygotes were indistinguishable from wild-type control mice, *Drg11*$^{-/-}$ mice had a very clear behavioral phenotype: they starved to death at weaning when handled with routine laboratory practice. Results of the present study, coupled with the observation that the incisors grew unchecked so as to protrude beyond the upper and lower lips, suggested that the post-weaning lethality reflected a deficit in feeding and/or drinking behaviors. When the incisors were periodically trimmed, and soft (moistened rodent chow; mash) or liquid (Liquidiet) food was made available *ad libitum* in an open-face shallow bowl, the *Drg11* mutants successfully ingested sufficient amounts of food to survive for up to 205 days postnatally. Their feeding behavior was grossly inefficient, however. Instead of licking the soft food, they used their paws to insert soft food into their mouths, and they usually failed in correctly targeting the mouth, therein covering themselves in the soft food. Low frequency head tremors came to accompany the attempts to insert the food-laden paw into their mouth. Consequently, *Drg11*$^{-/-}$ mice weighed significantly less than their wild-type counterparts (Fig. 6). Moreover, significantly reduced body weights were also displayed prior to weaning (Fig. 6), suggesting that suckling behaviors were also impacted by the *Drg11* mutation.

Discussion

The present study indicates that *Drg11* gene deletion results in a complete disintegration of the Me5 during embryonic development as a result of abnormal Me5 cell death. Consequently, muscle spindles disappear in the masticatory muscles, whereas the trigeminal motor nucleus and its peripheral projection appear to be grossly intact. Thus, *Drg11* is necessary for the development of the oro-facial proprioceptive first-order neurons. The latter may contribute to the inefficient ingestive behaviors of *Drg11*$^{-/-}$ mice and their resultant reduced body weights.

Drg11 is required for the survival of Me5 neurons

Drg11 was expressed in the Me5 during embryonic development and Me5 neurons were absent in *Drg11*$^{-/-}$ mice at birth. However, DiI labeling and Neo immunostaining showed that a normal complement of Me5 neurons exist in *Drg11*$^{-/-}$ embryos prior to E13.5,

suggesting that the initial generation, differentiation, migration and peripheral projections of the Me5 neurons are normal in the absence of *Drg11* expression. However, by E13.5, Me5 neurons were far less numerous reflecting excess cell death. The latter conclusion stems from the increased prevalence of caspase-3- and TUNEL-positive neurons in the Me5 of *Drg11*$^{-/-}$ embryos. The absence of Me5 neurons at birth is, therefore, most likely due to pervasive Me5 cell death in the absence of *Drg11* expression. Thus, *Drg11* is required for the survival of Me5 neurons during embryonic development.

Drg11 is also expressed in proprioceptive neurons in the DRG that convey proprioceptive sensation from the body to the central nervous system. The proprioceptive DRG neurons project to the motor neurons in the spinal ventral horn for the establishment of the monosynaptic stretch reflex circuit in the spinal cord, and they also project rostrally to the dorsal column nuclei. The dorsal column nuclei-projecting DRG neurons are the first-order neuron in the medial lemniscal pathway that conveys proprioceptive information from the body to the thalamus. While the ventral horn-projection is maintained in *Drg11*$^{-/-}$ mice (Chen et al., 2001), it will be of interest to determine whether the proprioceptive projection to the dorsal column nuclei is impacted in *Drg11*$^{-/-}$ mice. This would be interesting in light of the fact that the digit map in the dorsal column nuclei, thalamus and somatosensory cortex of *Drg11*$^{-/-}$ mice is intact (Ding et al., 2003). In this regard it is pertinent to note that *Drg11*$^{-/-}$ mice display normal locomotion (Chen et al., 2001) and normal number of proprioceptive neurons in the DRG (Chen et al., 2001; Rebelo et al 2006), indicating that the afferent pathway for proprioceptive inputs from the body is well maintained in *Drg11*$^{-/-}$ mice. Thus, *Drg11* appears to be differentially involved in the development of proprioceptive sensory neurons from the oro-facial region and the body. This may reflect a yet-to-be-determined consequence of their respective locations in the central and peripheral nervous systems.

It is also of interest that virtually all of the Me5 neurons disintegrate in *Drg11*$^{-/-}$ mice. Given that a relatively small percentage of the Me5 cells are not proprioceptors, but, rather, are periodontal mechanoreceptors (Capra and Wax, 1989), it would appear that *Drg11* is also necessary for the survival of at least some forms of mechanoreceptors. This conclusion is supported by recent studies of the postnatal *Drg11*$^{-/-}$ mice (Jacquin et al., submitted) indicating an approximately 60% neuronal survival in the trigeminal ganglion, as well as an approximately 50% and 70% respective cell survival in the trigeminal brainstem nuclei principalis and interpolaris, both of which are predominantly devoted to processing mechanoreceptive inputs.

Postnatal consequences of the Drg11 mutation

Drg11$^{+/-}$ mice are viable, fertile and apparently normal. However, a prior report indicated that the *Drg11*$^{-/-}$ mice are lethal at the time of weaning. The available data now strongly suggest that *Drg11*$^{-/-}$ mice lack a spinal and DRG nociceptive system (Chen et al., 2001; Rebelo et al., 2006), an orofacial proprioceptive system (present study), patterned representations of the whiskers in the lemniscal component of the whisker-barrel pathway (Ding et al., 2003), and approximately half of the normal numbers of cells in the trigeminal ganglion and in the trigeminal brainstem nuclei principalis and interpolaris (Jacquin et al.,

submitted). To what extent are the postnatal lethality and the above-listed neuronal phenotypes causally related? The most parsimonious explanation for the failure of postnatal *Drg11*$^{-/-}$ mice to thrive is that they have altered sensory control of the ingestive apparatus. It has been known for some time that trigeminal mechanosensory inputs are required for feeding and drinking behaviors in rodents (Jacquin and Zeigler, 1983) and that, prior to weaning, these inputs are necessary for suckling behaviors. Indeed, Hofer et al. (1981) demonstrated the bilateral infraorbital nerve transection at birth led to ineffective suckling behaviors and the pups soon died of starvation. Insofar as the *Drg11* mutation is, for all intents and purposes, an oro-facial mechanosensory "deafferentation", via the extensive loss of trigeminal ganglion and brainstem sensory nucleus neurons (Jacquin et al., submitted), this is sufficient cause for consequently altered ingestive behavior and reduced body weight. The presently described loss of proprioceptive afferent input from the masticatory muscles could further reduce the integrity of feeding behavior in *Drg11*$^{-/-}$ mice.

Experimental methods

Generation, maintenance, and genotyping of Drg11 mutant mice

Mice lacking *Drg11* expression were generated and genotyped as previously described (Chen et al., 2001). The plug date is considered embryonic day (E) 0.5. *Drg11*$^{+/-}$ mice were maintained in the mouse facility according to the protocols approved by the Committee of Use of Laboratory Animals and Common facility, Institute of Neuroscience, Chinese Academy of Sciences, China. *Drg11*$^{-/-}$ mice were usually sacrificed by the normal time of weaning. However, in 5 cases, attempts were made to sustain *Drg11*$^{-/-}$ mice post-weaning by providing a soft food diet, such as standard rodent lab chow dissolved in water. Their body weights and feeding behaviors were periodically assessed and compared to that of wild-type controls.

In situ hybridization, immunohistochemistry and TUNEL staining

In situ hybridization and immunohistochemistry were performed as previously described (Ding et al., 2003). The embryos and pups were perfused with 4% paraformaldehyde (PFA) in 0.01 M phosphate buffer saline (PBS; pH 7.2), and embryos younger than E14.5 were directly immersed in the fixative. After cryoprotection with 15% sucrose in PBS, the samples were frozen-sectioned and mounted onto frosted slides. An *in situ* hybridization RNA probe for *Drg11* was designed as the full length of its coding sequence. For immunostaining, the antibodies were diluted in PBS containing 1% bovine serum albumin and 0.3% Triton X-100. In the generation of *Drg11*$^{-/-}$ mice, neomycin phosphotransferase II (Neo) was fused to the third coding exon of the *Drg11* locus, and Neo expression resembles that of endogenous *Drg11* and, therefore, can be used to trace *Drg11* mutant cells (Chen et al., 2001; Ding et al., 2003). Tissue sections from *Drg11*$^{+/-}$ and *Drg11*$^{-/-}$ embryos were incubated with rabbit anti-Neo antibody (Cortex Biochem, San Leandro, CA, USA; 1:1000) overnight at 4°C. Other primary antibodies used in the present study were rabbit anti-parvalbumin (Swant, Bellinzona, Switzerland; 1:10,000), goat anti-calcitonin gene-related peptide (CGRP) (Biogenesis, Poole, UK; 1:1000), and rabbit anti-caspase-3 (Cell Signaling, Danvers, MA, USA; 1:500). After several rinses in PBS, the sections were incubated with biotinylated anti-rabbit or anti-goat IgG (Jackson Immuno Research, West

Grove, PA, USA; 1:200) for 1 hour at 37°C. The sections were then incubated with Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA; 1:1000), counterstained with Hoechst (Acros Organics, Morris Plains, NJ, USA) and observed under epifluorescent microscopy. Some sections were incubated with avidin-biotin-peroxidase complex (Elite Vectastain; Vector Laboratories, Burlingame, CA, USA) at 37°C for 30 minutes, followed by incubation with PBS containing 0.02% diaminobenzidine and 0.003% H_2O_2.

For TUNEL staining, embryos were cryoprotected in 15% sucrose overnight, and serial 12-μm-thick sections were cut on a cryostat. TUNEL staining was carried out by the use of previously described methods (Ding et al., 2003). Briefly, after blocking endogenous peroxidases by incubation in 10% methanol and 3% H_2O_2 in PBS for 30 minutes at room temperature, sections were incubated in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 10 minutes. The sections were incubated with terminal deoxynucleotidyl tranferase (TDT) buffer (Promega, Madison, WI, USA) for 5 minutes, and then with the TDT buffer containing 0.5 mM TDT (Promega) and 40 μM biotin-dUTP (Roche, Indianapolis, IN, USA) for 1 hour at 37°C. After several rinses with PBS, the sections were incubated with Cy3-conjugated streptavidin (Jackson ImmunoResearch) in PBS.

Tracing Studies

For labeling Me5 and Mo5 neurons at varied embryonic stages and P0, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) crystals (Molecular Probes) were inserted into the first branchial arch or jaw-closing muscles using fine forceps. The first branchial arch gives rise to the jaw-closing muscles during development. Tissues were kept in fixative for 3-4 days (E10.5-E12.5), 2 weeks (E13.5-E15.5), or 3-4 weeks (E16.5-E18.5, P0) at 37°C. Serial 200-μm-thick sections were cut transversely with a vibratome and labeling was observed under epifluorescent or laser confocal microscopy. For labeling the peripheral innervation of masticatory muscles, DiI crystals were inserted into the trigeminal mandibular nerve distal to the trigeminal ganglia. For labeling the Me5 and Mo5 neurons at postnatal stages, a solution of 2% cholera toxin B subunit (CTB) (Sigma, St. Louis, MO, USA) was injected into jaw-closing muscles through a micropipette. The mice were allowed to survive for 2 days, and perfused transcardially with 4% PFA. The brains were dissected out and processed for immunostaining with goat anti-CTB antibody (List Biological Laboratories, Campbell, CA, USA; 1:1000).

Data Analysis

At least four *Drg11*$^{-/-}$ and four wild-type controls were used for analysis of neuronal development at all ages studied. Each brain was cut into five sets of serial sections, and DiI-labeled, immunopositive or TUNEL-positive cells were viewed in at least one set of the sections. Sometimes, labeled cells were counted in every fifth section to gain a rough estimate of labeled cell prevalence. Total numbers of counted cells from one brain were pooled. Data are expressed as mean ± standard deviations, and analyzed by the use of student's *t* tests.

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References

Alley KE. Morphogenesis of the trigeminal mesencephalic nucleus in the hamster: cytogenesis andff neurone death. J. Embryol. Exp. Morphol. 1974; 31:99–121. [PubMed: 4819565]

Balice-Gordon RJ, Lichtman JW. In vivo observations of pre- and postsynaptic changes during the transition from multiple to single innervation at developing neuromuscular junctions. J. Neurosci. 1993; 13:834–855. [PubMed: 8426240]

Capra NF, Wax TD. Distribution and central projections of primary afferent neurons that innervate the masseter muscle and mandibular periodontium: a double-label study. J. Comp. Neurol. 1989; 279:341–352. [PubMed: 2465319]

Chen ZF, Rebelo S, White F, Malmberg AB, Baba H, Lima D, Woolf CJ, Basbaum AI, Anderson DJ. The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. Neuron. 2001; 31:59–73. [PubMed: 11498051]

De S, Nguyen AQ, Shuler CF, Turman JE Jr. Mesencephalic trigeminal nucleus development is dependent on Krox-20 expression. Dev. Neurosci. 2005; 27:49–58. [PubMed: 15886484]

Ding YQ, Yin J, Xu HM, Jacquin MF, Chen ZF. Formation of whisker-related principal sensory nucleus-based lemniscal pathway requires a paired homeodomain transcription factor. Drg11. J. Neurosci. 2003; 23:7246–7254.

Hofer MA, Fisher A, Shair H. Effects of infraorbital nerve section on survival, growth, and suckling behaviors of developing rats. J. Comp. Physiol. Psychol. 1981; 95:123–133. [PubMed: 7217407]

Ichikawa H, Qiu F, Xiang M, Sugimoto T. Brn-3a is required for the generation of proprioceptors in the mesencephalic trigeminal tract nucleus. Brain Res. 2005; 1053:203–206. [PubMed: 16040009]

Jacquin MF, Zeigler HP. Trigeminal orosensation and ingestive behavior in the rat. Behav. Neurosci. 1983; 97:62–97. [PubMed: 6838727]

Klaman LD, Boss O, Peroni OD, Kim JK, Martino JL, Zabolotny JM, Moghal N, Lubkin M, Kim Y-B, Sharpe AH, Stricker-Krongrad A, Shulman GI, Neel BG, Kahn BB. Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. Mol. Cell. Biol. 2000; 20:5479–5489. [PubMed: 10891488]

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science. 1997; 278:294–298. [PubMed: 9323209]

Lazarov NE. The mesencephalic trigeminal nucleus in the cat. Adv. Anat. Embryol. Cell. Biol. 2000; 153:1–103.

Lazarov NE. Comparative analysis of the chemical neuroanatomy of the mammalian trigeminal ganglion and mesencephalic trigeminal nucleus. Prog. Neurobiol. 2002; 66:19–59. [PubMed: 11897404]

Luo PF, Li JS. Monosynaptic connections between neurons of trigeminal mesencephalic nucleus and jaw-closing motoneurons in the rat: an intracellular horseradish peroxidase labelling study. Brain Res. 1991; 559:267–275. [PubMed: 1838952]

Luo PF, Wang BR, Peng ZZ, Li JS. Morphological characteristics and terminating patterns of masseteric neurons of the mesencephalic trigeminal nucleus in the rat: an intracellular horseradish peroxidase labeling study. J. Comp. Neurol. 1991; 303:286–299. [PubMed: 2013641]

Marani E, Usunoff KG. The trigeminal motonucleus in man. Arch. Physiol. Biochem. 1998; 106:346–354. [PubMed: 10441056]

Matsuo S, Ichikawa H, Silos-Santiago I, Arends JJ, Henderson TA, Kiyomiya K, Kurebe M, Jacquin MF. Proprioceptive afferents survive in the masseter muscle of trkC knockout mice. Neuroscience. 2000; 95:209–216. [PubMed: 10619477]

Narayanan CH, Narayanan Y. Determination of the embryonic origin of the mesencephalic nucleus of the trigeminal nerve in birds. J. Embryol. Exp. Morphol. 1978; 43:85–105. [PubMed: 632745]

Porter JD, Donaldson IM. The anatomical substrate for cat extraocular muscle proprioception. Neuroscience. 1991; 43:473–481. [PubMed: 1922779]

Rebelo S, Chen ZF, Anderson DJ, Lima D. Involvement of DRG11 in the development of the primary afferent nociceptive system. Mol. Cell. Neurosci. 2006; 33:236–246. [PubMed: 16978876]

Saito T, Greenwood A, Sun Q, Anderson DJ. Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. Mol. Cell. Neurosci. 1995; 6:280–292. [PubMed: 7496632]

Stainier DY, Bilder DH, Gilbert W. The B30 ganglioside is a cell surface marker for neural crest-derived neurons in the developing mouse. Dev. Biol. 1991; 144:177–188. [PubMed: 1995396]

Stainier DY, Gilbert W. Neuronal differentiation and maturation in the mouse trigeminal sensory system, in vivo and in vitro. J. Comp. Neurol. 1991; 311:300–312. [PubMed: 1753021]

Usunoff KG, Marani E, Schoen JH. The trigeminal system in man. Adv. Anat. Embryol. Cell. Biol. 1997; 136:1–126.

Xiang M, Gan L, Zhou L, Klein WH, Nathans J. Targeted deletion of the mouse POU domain gene Brn-3a causes selective loss of neurons in the brainstem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling. Proc. Natl. Acad. Sci. U.S.A. 1996; 93:11950–11955. [PubMed: 8876243]

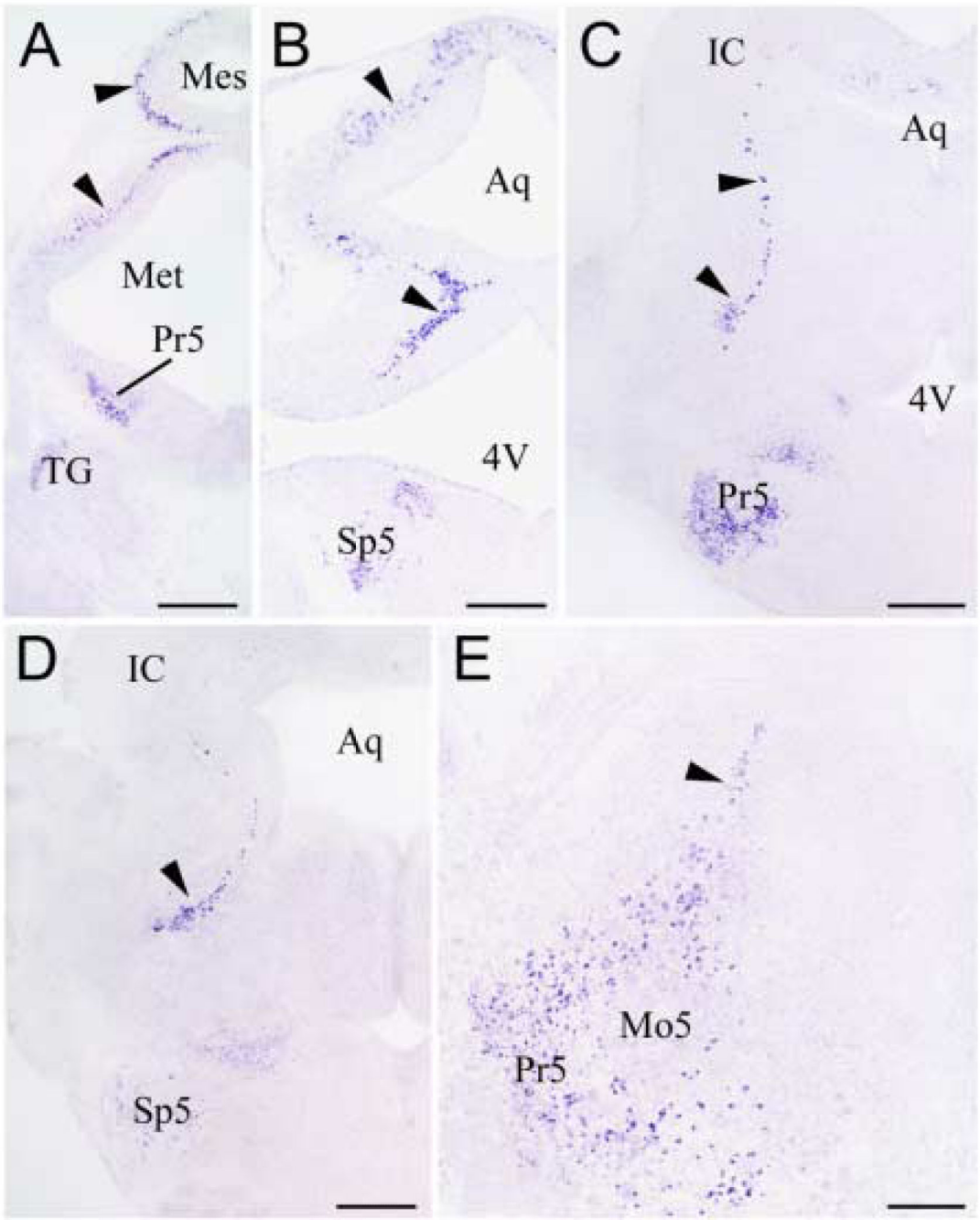

Fig. 1.

Expression of *Drg11* in the Me5 during embryonic and early postnatal development. (A) At E11.5, *Drg11* was detected in the presumptive Me5 (arrowheads) in the mesencephalon (Mes) and metencephalon (Met). Expression of *Drg11* was also seen in the principal sensory nucleus of the trigeminal nerve (Pr5) and trigeminal ganglion (TG), but not in the trigeminal motor nucleus (Mo5). (B) At E13.5, increased *Drg11*-expressing neurons (arrowheads) were detected in the Me5 and *Drg11* expression was also seen in the rostral subnucleus of the spinal trigeminal nucleus (Sp5). (C, D) *Drg11*-expressing neurons (arrowheads) were decreased in the Me5 at E15.5 (C) and E17.5 (D). (E) *Drg11* expression was observed in the Me5 (arrowhead) and Pr5, but not in the Mo5 at P7. 4V, fourth ventricle; Aq, aqueduct. Scale bars, 300 μm.

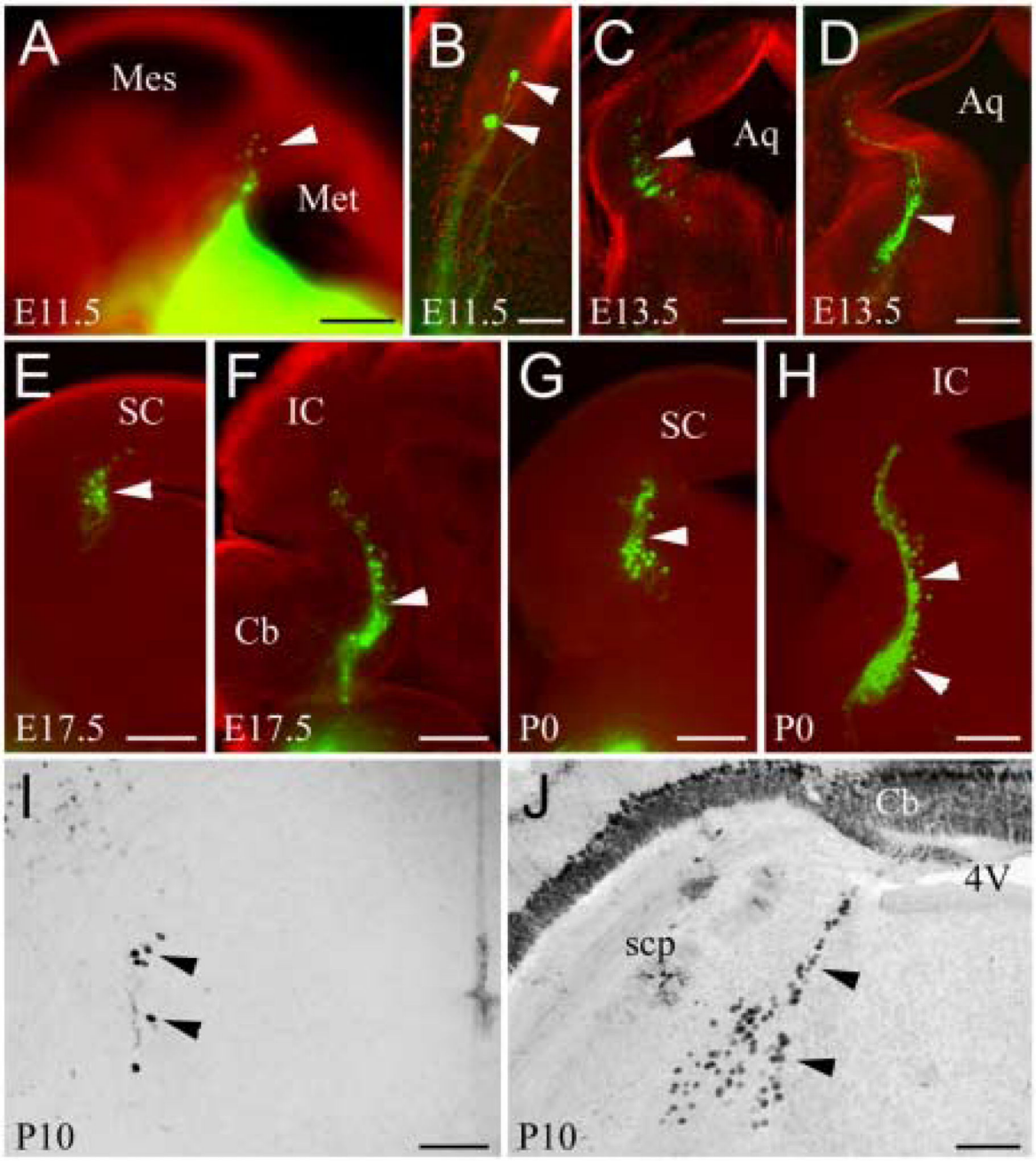

Fig. 2.

Development of the Me5 revealed by DiI labeling. Me5 neurons were labeled by DiI that was implanted into the first branchial arch or jaw-closing muscles. (A, B) At E11.5, a few DiI-labeled cells (green, pseudo color; arrowheads) were seen in the rostral metencephalon. DiI-labeled neurons in A were enlarged in B. (C, D) At E13.5, DiI-labeled Me5 neurons (arrowhead) were increased in both rostral Me5 (C) and caudal Me5 (D). (E-H) At E17.5 (E, F) and P0 (G, H), rostral Me5 in the midbrain (E, G) and caudal Me5 in the pons (F, H) were retrogradely labeled by DiI (arrowheads). (I, J) Me5 neurons (arrowheads) were positively immunostained with parvalbumin antibody in the midbrain (I) and the pons (J) at P10. The sections (A-H) were

counterstained with Hoechst (red, pseudo color). 4V, fourth ventricle; Aq, aqueduct; Cb, cerebellum; IC, inferior colliculus; SC, superior colliculus; scp, superior cerebellar peduncle. Scale bars, 400 μm.

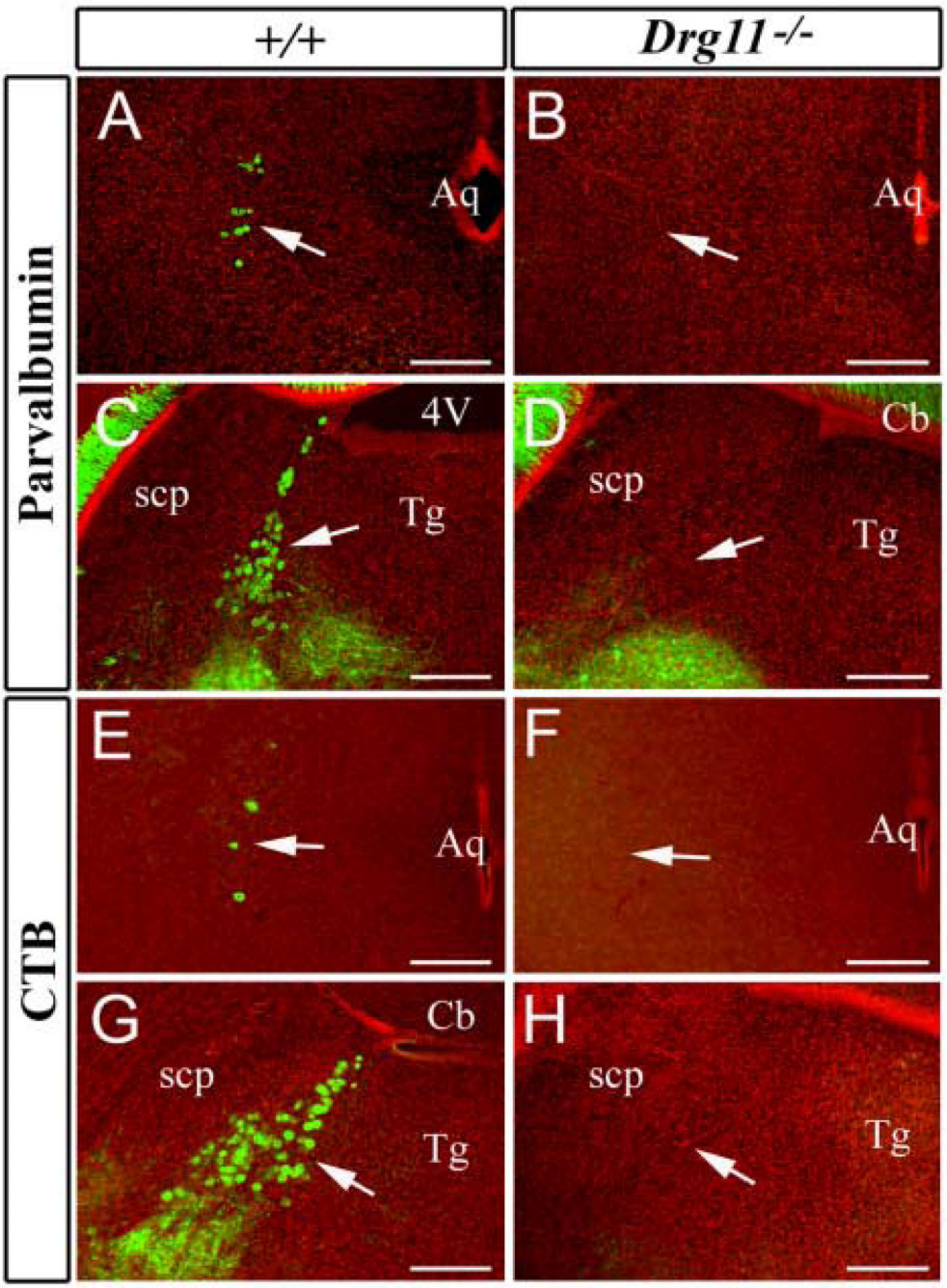

Fig. 3.

Loss of the Me5 in *Drg11*$^{-/-}$ mice. (A-D) Parvalbumin-positive neurons were observed in the rostral and caudal Me5 of wild-type mice (A, C), whereas no such positive neurons were detected in *Drg11*$^{-/-}$ mice at P15 (B, D). Arrow in (A, C) points to the parvalbumin-positive Me5 neurons, and arrow in (B, D) indicates the corresponding regions containing no positive neurons. (E-H) Cholera toxin B subunit (CTB)-labeled Me5 neurons in the midbrain (E) and pons (G) of wild-type mice, whereas no CTB-labeled neurons were observed in either rostral (F) or caudal (H) Me5 after the injection of CTB into jaw-closing muscles in *Drg11* mutant mice at P15. The sections were counterstained with Hoechst (red, pseudo color). 4V, fourth ventricle; Aq, aqueduct; Cb, cerebellum; scp, superior cerebellar peduncle; Tg, tegmentum. Scale bars, 600 μm.

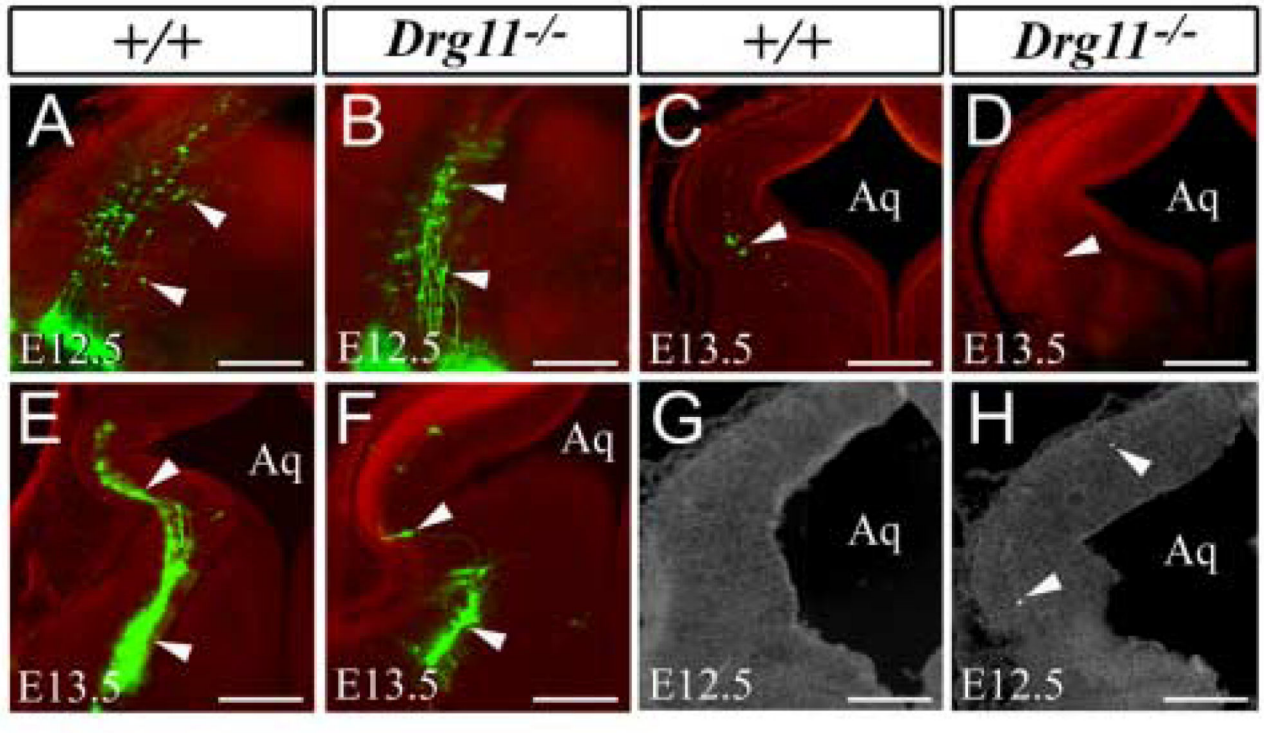

Fig. 4.

Decrease of Me5 neurons and increased cell apoptosis in the Me5 of *Drg11*$^{-/-}$ mice during development. (A, B) At E12.5, a similar number of DiI-labeled Me5 neurons (green, pseudo color; arrowheads) were observed in *Drg11*$^{-/-}$ embryo (B) compared with that of wild-type embryo (A) after the insertion of DiI crystals into jaw-closing muscles. (C-F) At E13.5, DiI-labeled neurons in the rostral Me5 were almost completely lost (arrowhead in D) and reduced dramatically in the caudal Me5 (arrowheads in F) of *Drg11*$^{-/-}$ mice compared with those of wild-type embryo (C, E). (G, H) At E12.5, more caspase-3-positive cells (arrowheads) were detected in the presumptive Me5 of *Drg11*$^{-/-}$ mice (H) compared with that of wild-type embryo (G). The sections (A-F) were counterstained with Hoechst (red, pseudo color). Aq, aqueduct. Scale bars, 500 μm for C-F; 400 μm for A, B, G and H.

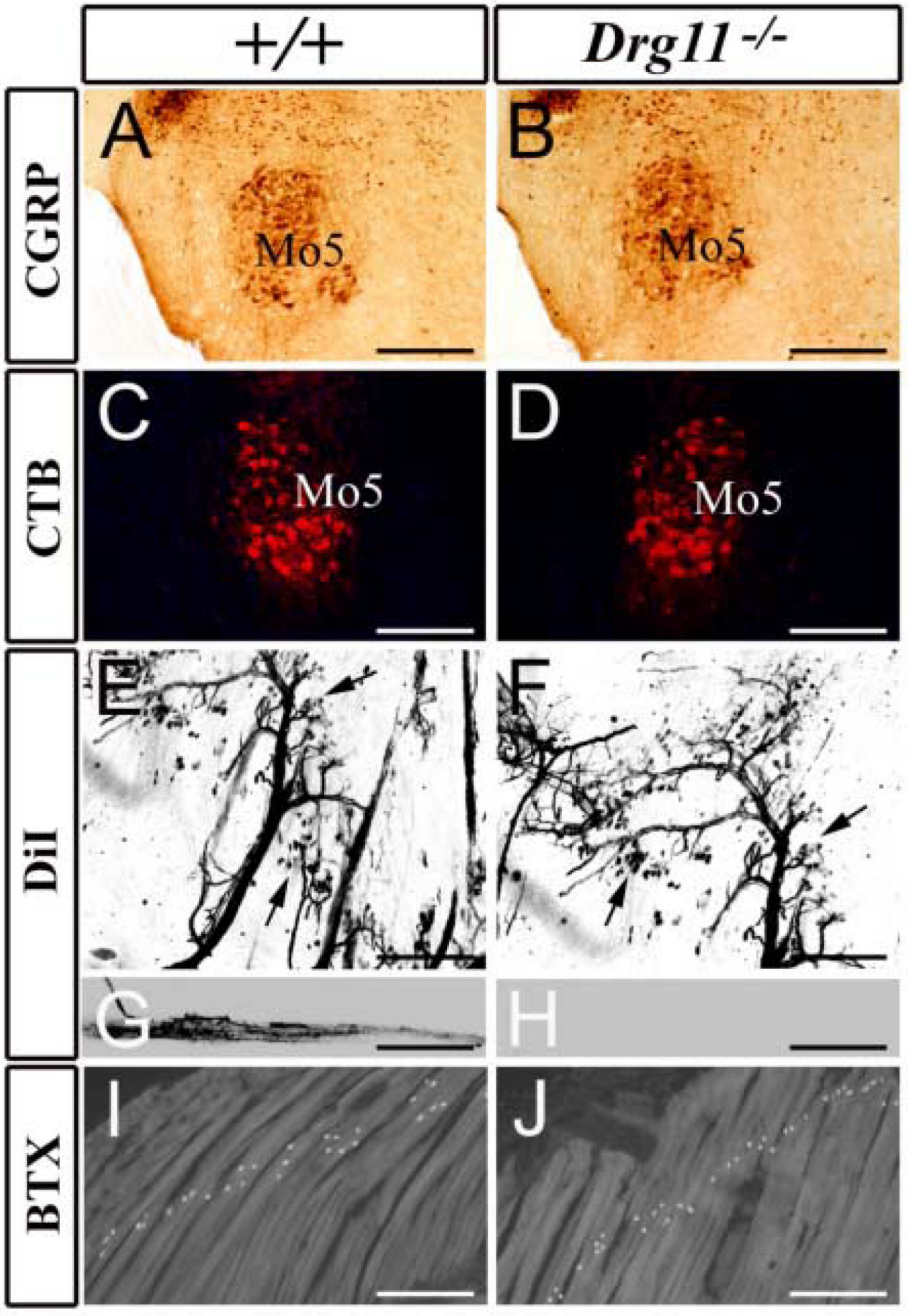

Fig. 5.

Peripheral innervation of trigeminal motor neurons in the masticatory muscles was maintained intact, whereas that of proprioceptive Me5 neurons was lost in *Drg11*$^{-/-}$ mice. (A, B), Trigeminal motor neurons in the Mo5 were calcitonin gene-related peptide (CGRP)-positive in both the wild-type (A) and *Drg11*$^{-/-}$ mice (B), and cell numbers were comparable between these two groups (A, B) at P16. (C, D) Cholera toxin B subunit (CTB)-labeled Mo5 neurons were present in both wild-type (C) and *Drg11*$^{-/-}$ mice (D) after the injection of CTB into jaw-closing muscles at P16. (E, F) Efferent innervation of masticatory muscle by trigeminal motor neurons was normal in the *Drg11*$^{-/-}$ mice (F) compared with that of wild-type mice (E) at P16. Arrows point to motor endplates in muscle fibers. (G, H) Primary sensory endings (i.e. muscle spindles) terminated in the

equatorial region of the intrafusal muscle fiber were observed in wild-type mice (G), whereas no such nerve endings were detected in *Drg11*$^{-/-}$ mice (H) at P16. (I, J) Clusters of postsynaptic acetylcholine receptors in masticatory muscle were detected in *Drg11*$^{-/-}$ mice (J) by α-bungarotoxin (BTX) labeling, and they were very similar to those in wild-type mice at P16 (I). Scale bars, 600 μm for A-F, I and J; 150 μm for G and H.

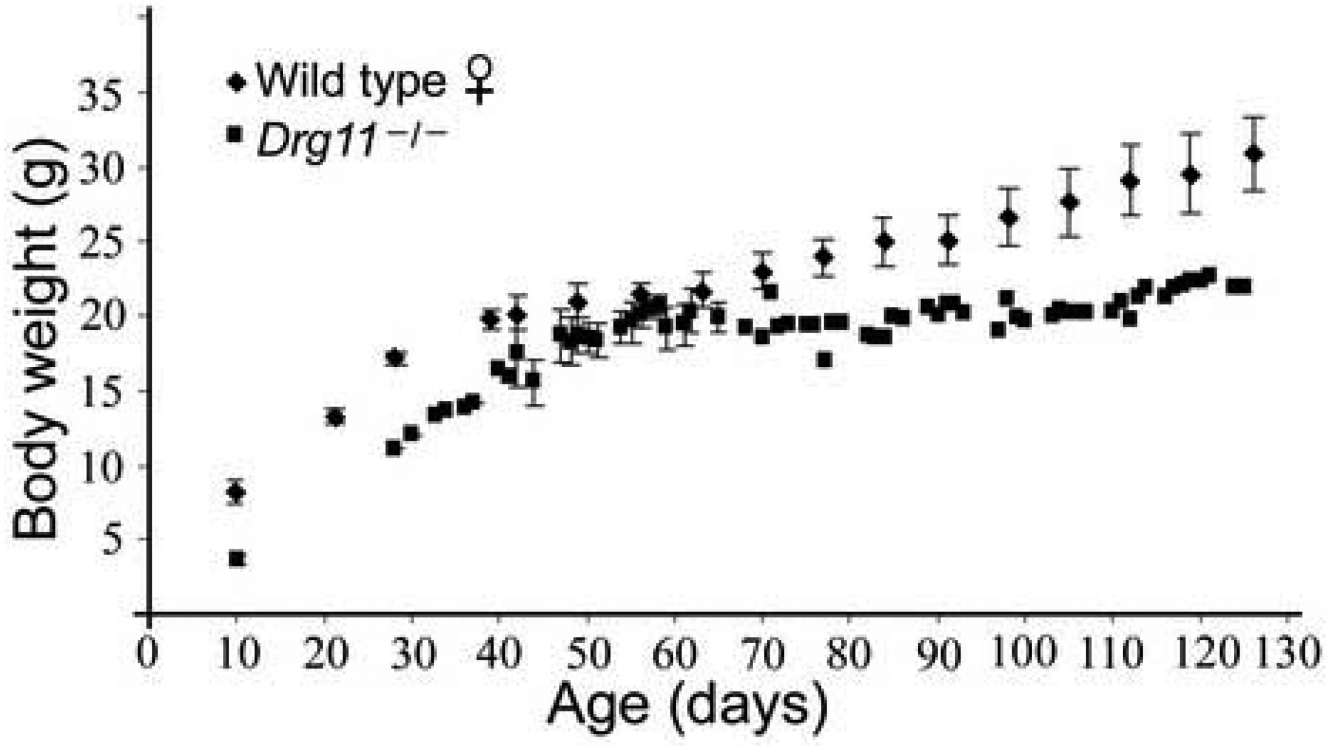

Fig. 6.

Body weights of *Drg11*$^{-/-}$ and wild-type mice at selected postnatal timepoints (mean ± standard deviation). *Drg11*$^{-/-}$ mice were weaned at postnatal day (P) 28 and provided a soft-food diet. When the mutant mice were offered Liquidiet, they could survive till P205. *Drg11*$^{-/-}$ mice are of unknown gender (at P10, n = 6; at P28-65, n = 4; at >P65, n = 1). Wild-type data from P21 and older are from female mice weaned on P21 and maintained on rodent lab chow; reproduced from Klaman et al. (2000).