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Role of motoneuron-derived neurotrophin 3 in survival and axonal projection of sensory neurons during neural circuit formation

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SUMMARY

Sensory neurons possess the central and peripheral branches and they form unique spinal neural circuits with motoneurons during development. Peripheral branches of sensory axons fasciculate with the motor axons that extend toward the peripheral muscles from the central nervous system (CNS), whereas the central branches of proprioceptive sensory neurons directly innervate motoneurons. Although anatomically well documented, the molecular mechanism underlying sensory-motor interaction during neural circuit formation is not fully understood. To investigate the role of motoneuron on sensory neuron development, we analyzed sensory neuron phenotypes in the dorsal root ganglia (DRG) of *Olig2* knockout (KO) mouse embryos, which lack motoneurons. We found an increased number of apoptotic cells in the DRG of *Olig2* KO embryos at embryonic day (E) 10.5. Furthermore, abnormal axonal projections of sensory neurons were observed in both the peripheral branches at E10.5 and central branches at E15.5. To understand the motoneuron-derived factor that regulates sensory neuron development, we focused on neurotrophin 3 (Ntf3; NT-3), because *Ntf3* and its receptors (*Trk*) are strongly expressed in motoneurons and sensory neurons, respectively. The significance of motoneuron-derived Ntf3 was analyzed using *Ntf3* conditional knockout (cKO) embryos, in which we observed increased apoptosis and abnormal projection of the central branch innervating motoneuron, the phenotypes being apparently comparable with that of *Olig2* KO embryos. Taken together, we show that the motoneuron is a functional source of Ntf3 and motoneuron-derived Ntf3 is an essential pre-target neurotrophin for survival and axonal projection of sensory neurons.

KEY WORDS: Motor and sensory circuits, Neurotrophin 3, Ntf3, NT-3, Apoptosis, Axonal projection, Pre-target neurotrophin, Proprioceptive neuron

INTRODUCTION

Neural circuit formation requires multiple steps: cell type specification, migration, axonal projection to their appropriate targets, formation of functional synapses and elimination of excess cells and synapses. Neural crest-derived sensory neuron precursors originate within the tips of neural folds and migrate ventrally to form the dorsal root ganglion (DRG). Sensory neurons extend two axon branches: the peripheral branch and the central branch. Different types of sensory neurons are specialized for different perceptual modalities. For example, proprioceptive sensory neurons extend axons to alpha-motoneurons in the ventral horn, and provide information about muscle length and tension to the spinal cord. This sensory-motor circuit provides an excellent system with which to study neural circuit formation. The developmental molecular interaction between motoneurons and sensory neurons has just begun to be elucidated (Gallarda et al., 2008; Wang et al., 2011). For example, Sema3e and its high-affinity receptor plexin D1 (Plxnd1), which are expressed by selected motoneuron pools and proprioceptive sensory neurons, respectively, are crucial determinants of synaptic specificity in sensory-motor circuits (Pecho-Vrieseling et al., 2009). It is known that early-born neurons regulate late-born neurons during development of some neuronal circuits, such as the olfactory pathway (Takeuchi et al., 2010) and the thalamocortical pathway (McConnell et al., 1989). As motoneuron development precedes sensory neuron development (Wentworth, 1984; Landmesser and Honig, 1986; Wang et al., 2011), it is conceivable that motoneurons have substantial roles in sensory neuron development.

In order to investigate the role of motoneurons on early sensory neuron development, we analyzed sensory neuron phenotypes in the DRG of *Olig2* knockout (KO) embryos, which have no motoneuron in the spinal cord. Olig2 is a basic helix-loop-helix (bHLH) transcription factor that is essential for motoneuron and oligodendrocyte development (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Here, we report abnormal phenotypes in the sensory neurons of *Olig2* KO mice: first, the decreased number of sensory neurons and the increased number of apoptotic cells in the DRG; and second, the abnormal axonal projection of central and peripheral branches of DRG neurons. The neurotrophic factor neurotrophin 3 (Ntf3; previously NT-3) is specifically and strongly expressed in the motoneuron column of the embryonic spinal cord (Ernfors and Persson, 1991). As Ntf3 is not expressed in the ventral spinal cord of *Olig2* KO embryos, we

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investigated the role of motoneuron-derived Ntf3 in suppression of apoptosis and in proper axonal projection of sensory neurons during development.

Ntf3 is one of the well-known neurotrophic factors (Chao, 2003), which has been shown to exert a strong survival activity on DRG neurons (EIShamy and Ernfors, 1996; Fariñas et al., 1994; Liebl et al., 1997; Tojo et al., 1995) and it acts as a chemoattractant for DRG axons (Genc et al., 2004; Tucker et al., 2001). Main sources of Ntf3 are thought to be the target of innervation or the mesenchyme (Ernfors, 2001; Patapoutian et al., 1999). Although Ntf3 has been suggested to act as a pre-target neurotrophin, its detailed source and function have not yet been elucidated (Gaese et al., 1994; Howe and Mobley, 2005). Here, we generated and analyzed *Ntf3* conditional KO (cKO) mice, in which the *Ntf3* gene is deleted in the ventral spinal cord. In these mice, we observed increased cell death of sensory neurons and abnormal axonal projection of central branches, the phenotypes being comparable with those of *Olig2* KO mice. Our data indicate that motoneuron is a functional source of Ntf3 and that motoneuron-derived Ntf3 is involved in early sensory-motor interaction by acting as a pre-target neurotrophin for early sensory neurons. Taken together, this study shows the importance of a motoneuron-derived factor in the sensory-motor circuit formation.

MATERIALS AND METHODS

Mice

We used an *Olig2-CreER* mouse (Takebayashi et al., 2002), an *Olig2-Cre* knock-in mouse (H.T., unpublished) and a *Pc36* transgenic mouse, which have ubiquitous Cre activity (Ding et al., 2005); a flox *Ntf3* mouse (Bates et al., 1999; obtained from Jackson Laboratory; stock number 003541); and a *Z/EG* reporter mouse (Novak et al., 2000). The *Olig2* mice used in this study were generated using an *Olig2-CreER* allele. PCR genotyping of *Olig2*, *Cre* and *Z/EG* alleles was performed as described previously (Masahira et al., 2006; Takebayashi et al., 2002; Tsujita et al., 1999). The *Ntf3* null allele was generated by mating a flox *Ntf3* mouse line with a *Pc36* Cre driver line. PCR primers (*Ntf3*_null_S2, 5′-TCA GGG GCT GGG CGA GGA AA-3′; *Ntf3*_null_AS3, 5′-AGT TCT TTG GGG GAG GGG GCA-3′) were designed to detect the recombined flox *Ntf3* allele, the *Ntf3* null allele and the wild-type *Ntf3* allele, which yielded 2.5 kb, 1.3 kb and 2.3 kb products, respectively, using KOD FX Neo Taq (TOYOBO) (supplementary material Fig. S5). PCR genotyping for detection of the wild-type *Ntf3* allele (375 bp band) and the mutant alleles (either flox *Ntf3* allele or *Ntf3* null allele, 325 bp band) was performed according to the protocol from Jackson Laboratory using following primers (oIMR1336, 5′-GGA AGC AAG CAA TCA GAA GAC C-3′; oIMR1337, 5′-GAA TTG AGA GAG TCT TCC GGC-3′) and Quick Taq HS (TOYOBO). We maintained *Olig2-CreER* mice, *Olig2-Cre* mice and *Z/EG* mice in the ICR background (Japan SLC). For embryo staging, the day of detection of vaginal plug was considered to be E0.5. All procedures were approved by the Animal Research Committees at Kumamoto University and National Institute for Physiological Sciences.

Chick and in ovo electroporation

Fertilized hens' eggs were purchased locally and incubated at 38°C. Approximately 1 μl of the plasmid DNA (1 μg/μl: pCAG-GFP and pCAG-DsRed, or pCAG-Ntf3-GFP and pCAG-DsRed) was microinjected into the chick neural tube at E3 [Hamburger and Hamilton (HH) stage 18-20] (Hamburger and Hamilton, 1951) with a glass capillary and then electroporated using an electroporator (CUY-21, NEPA GENE; 25 V, 30 mseconds on, 100 mseconds off, six pulses) and a platinum electrode (CUY610P4-1, NEPA GENE). After 48 hours of electroporation (at E5, HH stage 25-26), the electroporated embryos were fixed overnight using 4% paraformaldehyde (PFA) to carry out immunohistochemical analyses. The electroporated areas were identified by DsRed fluorescence.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Takebayashi et al., 2008). Cryosections (20 μm thick) were stained with following primary antibodies: rabbit anti-cleaved caspase 3 (1:500, Cell Signaling Technology), rabbit anti-TrkA (1:2000; a gift from Dr Reichardt, UCSF, CA, USA), rabbit anti-TrkC (1:200, Santa Cruz Biotechnology), rabbit anti-Olig2 (1:200, IBL; 1:500, Chemicon), rat anti-Ki67 (1:20, TEC-3, DakoCytomation), mouse anti-ßIII-tubulin (1:1000; Tuj1, Covance), mouse anti-HB9 (1:20, MNR2 supernatant, DSHB), mouse anti-Islet1/2 (1:20, 39.4D5 supernatant, DSHB), mouse anti-neurofilament M (1:20, 1C8; Watanabe et al., 2006) and chicken anti-peripherin (1:1000; Aves Labs). For fluorescence immunostaining, species-specific antibodies conjugated to Alexa 488 or Alexa 594 (1:2000; Invitrogen/Molecular Probes) were applied and nuclei were counterstained with Hoechst 33342 (1 μg/ml; Sigma). For diaminobenzidine (DAB) staining, biotinylated species-specific goat antibodies (1:400; Millipore), or a biotinylated donkey anti-chick antibody (1:100; Jackson ImmunoResearch) was applied and then processed with the ABC method (ABC Elite kit, Vector Laboratories) following the manufacturer's instructions. The images were captured using an Olympus microscope and digital camera system (BX51 and DP70, or BX53 and DP72; Olympus).

In situ hybridization

In situ hybridization was performed as described previously (Takebayashi et al., 2008) using following riboprobes: rat *TrkA* (*Ntrk1*, Matsumoto et al., 2001), mouse *TrkB* (*Ntrk2*, Singh et al., 1997), rat *TrkC* (*Ntrk3*, Matsumoto et al., 2001), mouse *Ngf* (Yoshida et al., 2001), mouse *Bdnf* (Singh et al., 1997), mouse *Ntf3* (GenBank Accession Number NM_008742, nt 280-987), mouse *Runx1* (GenBank Accession Number NM_001111023, nt 2724-3861), mouse *Runx3* (GenBank Accession Number NM_019732, nt 944-1938) and mouse *Olig2* (Takebayashi et al., 2000). Digoxigenin (DIG)-labeled riboprobes were synthesized from the plasmids using in vitro transcription. Cryosections (20 μm) were fixed in 4% PFA, treated with Proteinase K (1 μg/ml), acetylated and then hybridized with DIG-labeled riboprobes overnight at 65°C. Then, sections were washed, blocked with blocking buffer for 1 hour at room temperature and incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (Roche) in blocking buffer. Alkaline phosphatase was visualized by nitro-blue tetrazolium chloride (NBT, Roche) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP, Roche) according to the manufacturer's instructions.

Statistical analysis

Quantification of *TrkB*-positive, *TrkC*-positive, *Runx3*-positive, cleaved caspase 3-positive and Ki67-positive cells was performed in E10.5, E13.5 and E18.5 transverse sections at thoracic levels. Cells with positive staining were manually counted on 10-20 sections from three or four embryos for each condition. Statistical significance was determined using Student's *t*-test.

RESULTS

Sensory cell death and reduced cell number in the DRG of *Olig2* KO embryos

To investigate the role of motoneurons on sensory neuron development, DRG phenotypes were analyzed in *Olig2* KO embryos, which have no motoneuron in the spinal cord (supplementary material Fig. S1). The specification of DRG neurons occurs at a later stage (after E11.5), which lead to the subtype-specific expression of each Trk receptor (supplementary material Fig. S4D-F) (Marmigère and Ernfors, 2007). As most DRG neuron progenitors express all *Trk* receptors at E10.5, we performed in situ hybridization at E13.5 and E18.5 (Marmigère and Ernfors, 2007) in order to analyze the number of nociceptive (*TrkA* positive; Ntrk1 – Mouse Genome Informatics), mechanoreceptive (*TrkB* positive; Ntrk2 – Mouse Genome Informatics) and proprioceptive (*TrkC* positive; Ntrk3 – Mouse Genome Informatics) sensory neurons. Although quantification of nociceptive (*TrkA*-positive) neurons was difficult owing to the presence of a large number of positive cells in the DRG, no remarkable change was observed between

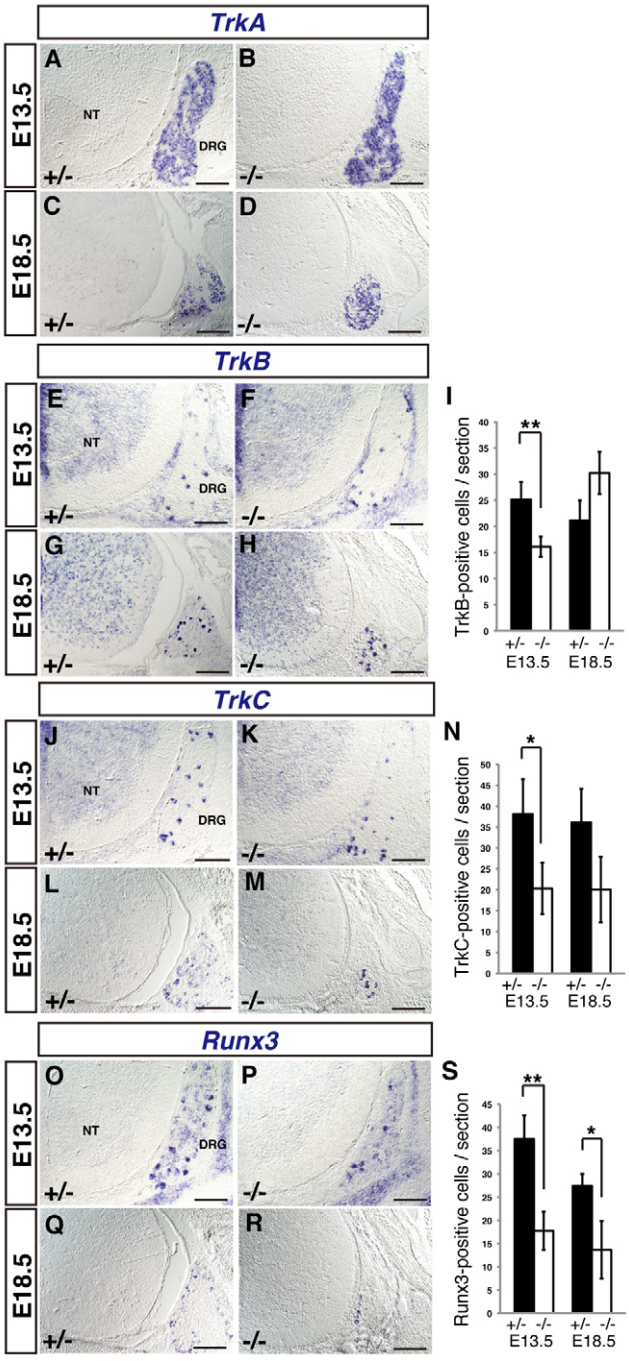

Fig. 1. Reduced number of sensory neurons in the DRG of *Olig2* KO mice. (**A-D**) Differential interference contrast (DIC) micrographs of *TrkA* in situ hybridization in the transverse sections of *Olig2* heterozygous control embryos at E13.5 (A) and E18.5 (C), and *Olig2* homozygous embryos at E13.5 (B) and E18.5 (D). (**E-H**) *TrkB* in situ hybridization in *Olig2* heterozygotes at E13.5 (E) and E18.5 (G), and *Olig2* homozygotes at E13.5 (F) and E18.5 (H). The number of *TrkB*-positive cells was reduced in the DRG of *Olig2* KO mice at E13.5. (**I**) Quantitative analysis of *TrkB*-positive cells in the DRG at E13.5 and E18.5. Values are mean±s.d. (**P<0.01, *t*-test). (**J-M**) *TrkC* in situ hybridization in *Olig2* heterozygotes at E13.5 (J) and E18.5 (L), and *Olig2* homozygotes at E13.5 (K) and E18.5 (M). *TrkC*-positive cells were also reduced in *Olig2* KO mice at E13.5. (**N**) Quantitative analysis of *TrkC*-positive cells in the DRG at E13.5 and E18.5. Values are mean±s.d. (*P<0.05, *t*-test). (**O-R**) *Runx3* in situ hybridization in *Olig2* heterozygotes at E13.5 (O) and at E18.5 (Q), and *Olig2* homozygotes at E13.5 (P) and at E18.5 (R). In addition, *Runx3*-positive cells were reduced in *Olig2* KO mice both at E13.5 and E18.5. *Runx3* is a proprioceptive neuron marker (Nakamura et al., 2008). (**S**) Quantitative analysis of *Runx3*-positive cells in the DRG at E13.5 and E18.5. DRG, dorsal root ganglion. NT, neural tube. Values are mean±s.d. (**P<0.01, *P<0.05, *t*-test). Scale bars: 50 μm in A,B,E,F,J,K,O,P; 100 μm in C,D,G,H,L,M,Q,R.

heterozygotes and *Olig2* KO mice (Fig. 1A-D). The number of mechanoreceptive (*TrkB*-positive) neurons in the DRG of *Olig2* KO mice was decreased significantly at E13.5 (P<0.01), but the change was not significant at E18.5 (Fig. 1E-I). Furthermore, the number of *TrkC*-positive and *Runx3*-positive proprioceptive neurons was decreased at E13.5 (Fig. 1J,K,N-P,S). We observed similar phenomena at E18.5 (Fig. 1L-N), but only the decrease of *Runx3*-positive proprioceptive neurons was statistically significant (Fig. 1Q-S). Therefore, in *Olig2* KO embryos, at least the number of mechanoreceptive and proprioceptive neurons was reduced at E13.5. This abnormal population of sensory neurons observed in *Olig2* KO embryos results from a cell non-autonomous effect of Olig2 because no Olig2 expression is observed in the developing DRG of wild-type embryos at E9.5 and E10.5 (supplementary material Fig. S2).

We next analyzed cell death and cell proliferation to investigate why DRG neurons are decreased in *Olig2* KO mice. An increased number of cleaved caspase 3-positive apoptotic cells was observed in the DRG of *Olig2* KO mice at E10.5 (Fig. 2A,B,E) (Takebayashi et al., 2008). At E13.5, the difference between heterozygotes and *Olig2* KO mice was not statistically significant (Fig. 2C-E). On the other hand, the number of Ki67-positive proliferative cells was not changed significantly in the DRG either at E10.5 or at E13.5 (Fig. 2F-J). These data indicate that the decrease in the number of DRG neuron is due to the increased cell death, but not due to the decreased proliferation.

Abnormal axonal projection of sensory neurons in the *Olig2* KO mice

We next analyzed axonal projections of DRG neurons in *Olig2* KO mice using βIII-tubulin immunostaining. In *Olig2* KO mice, there are no motoneuron axons because of the lack of motoneurons (Fig. 3A-D). In addition, an abnormal axonal projection of the peripheral branches of sensory neurons was observed both at E10.5 and at E12.5 in those mutants (Fig. 3A-D). This phenotype was confirmed by whole-mount immunohistochemistry using a neurofilament M antibody at E10.5, and the results showed an irregular sensory axonal projection and fasciculation (supplementary material Fig. S3). Next, we performed immunostaining for neurofilament M or peripherin at E15.5. The Ia afferents, which constitute the central branches of proprioceptive neurons, are projected to motoneurons in the heterozygous spinal cord (Fig. 3E,G,I,K, arrows) (Ozaki and Snider, 1997). In *Olig2* KO mice, the axonal projection of Ia afferents did not reach the ventral spinal cord (Fig. 3F,H,J,L, arrows).

Motoneuron-derived Ntf3 is an important factor for sensory neuron development

We hypothesized that motoneuron-derived neurotrophic factors are important for survival of sensory neurons, because the most prominent phenotype of the E10.5 *Olig2* KO spinal cord is motoneuron deficiency. Among the neurotrophic factors, we

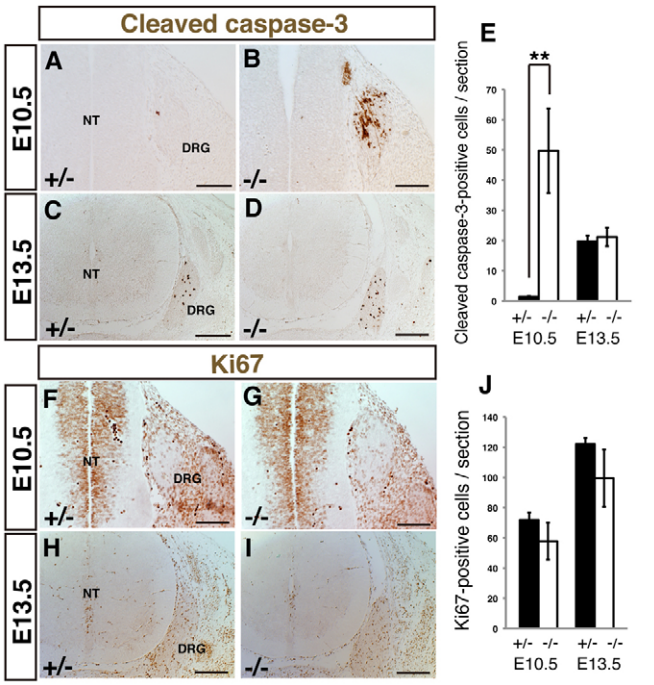

Fig. 2. Increased number of apoptotic cells in the DRG of *Olig2* KO mice. (**A-D**) DIC micrographs of cleaved caspase 3 immunostaining in the transverse sections of *Olig2* heterozygotes at E10.5 (A) and at E13.5 (C), and *Olig2* homozygotes at E10.5 (B) and E13.5 (D). (**E**) Quantitative analysis of cleaved caspase 3-positive cells in the DRG at E10.5 and E13.5. Values are mean±s.d. (**P<0.01, t-test). (**F-I**) Ki67 immunostaining in *Olig2* heterozygotes at E10.5 (F) and E13.5 (H), and *Olig2* homozygotes at E10.5 (G) and E13.5 (I). (**J**) Quantitative analysis of Ki67-positive cells in the DRG at E10.5 and E13.5. There was no significant difference in the number of Ki67-positive cells at E10.5 and E13.5. DRG, dorsal root ganglion. Scale bars: 50 μm in A,B,F,G; 100 μm in C,D,H,I.

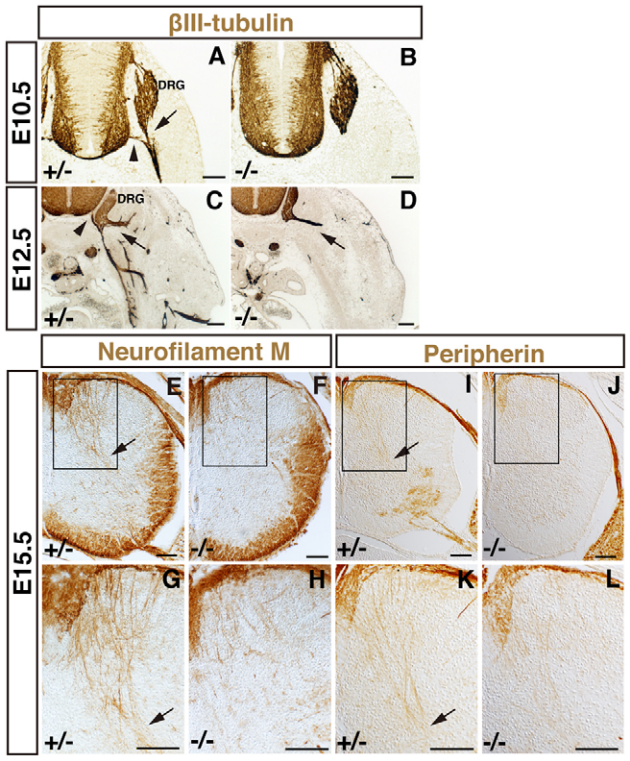

Fig. 3. Abnormal axonal projection of DRG neurons in *Olig2* KO mice. (**A-D**) DIC micrographs of βIII-tubulin immunostaining in the transverse sections of *Olig2* heterozygotes at E10.5 (A) and at E12.5 (C), and *Olig2* homozygotes at E10.5 (B) and E12.5 (D). Arrowheads and arrows indicate motoneuron axons and peripheral branches of sensory neuron, respectively. DRG, dorsal root ganglion. (**E**,**F**) Neurofilament M immunostaining in *Olig2* heterozygotes at E15.5 (E) and *Olig2* homozygotes at E15.5 (F). (**G**,**H**) Higher magnification views of boxed areas in E,F, respectively. (**I**,**J**) Peripherin immunostaining in *Olig2* heterozygotes at E15.5 (I) and *Olig2* homozygotes at E15.5 (J). (**K**,**L**) Higher magnification views of boxed areas in I,J, respectively. Note the absence of the stained fibers projecting to the ventral horn (arrows) in the spinal cord of *Olig2* KO embryos. Scale bars: 100 μm.

focused on *Ntf3*, because it is specifically and strongly expressed in the motor column within the ventral spinal cord of heterozygote embryos at E10.5 and E13.5 (Fig. 4A,C) (Ernfors and Persson, 1991; Fariñas et al., 1996) and *Ntf3* expression in the *Olig2* KO spinal cord is below detection level (Fig. 4B,D). We also performed intensive expression analyses of two other neurotrophic factors (*Ngf* and *Bdnf*) in motoneurons and their receptors (*TrkA*, *TrkB* and *TrkC*) in the DRG. We confirmed that the expression of *Ngf* and *Bdnf* is below detection levels at E10.5 (supplementary material Fig. S4A,B). The *Ntf3* expression in motoneurons became weaker during development, whereas the expression outside the spinal cord, such as in muscle and in skin, becomes stronger (Fig. 4A-D, arrowheads). The expression patterns of *Ntf3* in the motoneuron and *TrkC* receptor in the DRG (supplementary material Fig. S4F) suggested that motoneuron-derived Ntf3 had an important role in the sensory neuron development at the early stage (neurogenic stage, ~E10) and that mesenchyme- or target-derived Ntf3 takes over the role at the late stage (gliogenic stage, ~E13). To investigate the role of motoneuron-derived Ntf3, we generated *Ntf3* cKO mice by crossing *Olig2-Cre*; *Ntf3* null male mice and flox *Ntf3* female mice (supplementary material Fig. S5). As expected, no *Ntf3* expression was observed in the motor column of the *Olig2-Cre*; *Ntf3* cKO mice (Fig. 5A,B). Interestingly, the number of cleaved caspase 3-positive cells was dramatically increased in the DRG of *Ntf3* cKO mice at E10.5, compared with that of control embryos (Fig. 5C-E). The number of both *TrkC*-positive and *Runx3*-positive proprioceptive neurons was decreased in the DRG of *Ntf3* cKO mice at E13.5 (Fig. 5F-J). Peripheral branches seemed to be normal in *Ntf3* cKO mice at E10.5, as observed by βIII-tubulin immunohistochemistry (supplementary material Fig. S6). The immunostaining for neurofilament M and peripherin showed that the Ia afferents did not reach motoneurons (Fig. 5K-R). These data demonstrate that motoneruon-derived Ntf3 is essential for sensory neuron survival and proper innervation of Ia afferents.

Ntf3 secretion from motoneurons

Finally, we addressed the mode of Ntf3 secretion from motoneurons. As Ntf3 is believed to be a local neurotrophin (ElShamy and Ernfors, 1996) and sensory neuron axons have close contact with motoneuron axons in the spinal nerve at E10.5, transaxonal secretion is one suggested mechanism. To confirm the localization of Ntf3-GFP protein in the motoneuron axons in vivo, we next performed in ovo electroporation into chick embryonic spinal cord at E3 (HH stage 18-20) using DsRed as a control to indicate cytoplasmic localization. Indeed, we observed Ntf3-GFP localization in the motoneuron axons in vivo at E5 (HH stage 25-26, Fig. 6D-F). We also noticed that Ntf3-GFP signals were

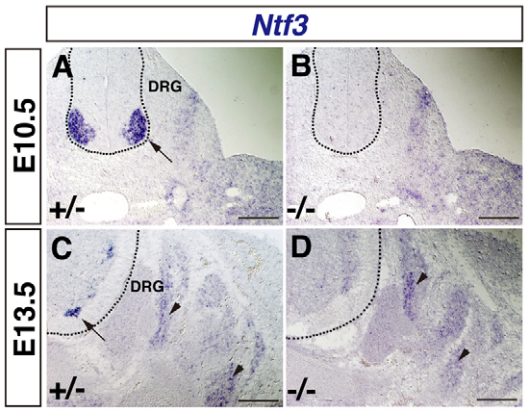

Fig. 4. *Ntf3* expression in embryonic motoneurons. (**A-D**) DIC micrographs of *Ntf3* in situ hybridization in the transverse sections of *Olig2* heterozygotes at E10.5 (A), *Olig2* homozygotes at E10.5 (B), *Olig2* heterozygotes at E13.5 (C) and *Olig2* homozygotes at E13.5 (D). *Ntf3* expression was observed in the *Olig2* heterozygous spinal cord, but not in the *Olig2* homozygous spinal cord, which indicate specific *Ntf3* expression in the motoneurons. Dotted lines indicate margins of the spinal cord. Arrows indicate *Ntf3* expression in the motoneurons, which are absent in the spinal cord of *Olig2* homozygous embryos. Arrowheads indicate *Ntf3* expression in the mesenchyme. DRG, dorsal root ganglion. Scale bars: 100 μm.

observed in the DRG and the mesenchyme surrounding the spinal cord (Fig. 6D,F arrowheads; supplementary material Fig. S7B), whereas GFP signals were observed only intracellularly in the control experiment (Fig. 6A-C; supplementary material Fig. S7A), suggesting Ntf3-GFP secretion from electroporated cells in the neural tube. These data suggest that the motoneuron-secreted Ntf3 reaches the DRG not only from motor axons, but also from the margin of the spinal cord.

DISCUSSION

In this study, we analyzed the DRG phenotype of motoneuron-deficient *Olig2* KO mice to investigate the role of motoneurons on sensory neuron development, and demonstrated increased apoptosis and abnormal projection of peripheral and central branches of sensory axons. The neurotrophic factor *Ntf3* is specifically and strongly expressed in the motoneuron column of the embryonic spinal cord (Fig. 4A,C) (Ernfors and Persson, 1991). As *Ntf3* expression was not observed in the ventral spinal cord of *Olig2* KO mice, we analyzed the role of motoneuron-derived Ntf3 on sensory neuron development by generating *Ntf3* cKO mice. We further demonstrated that the motoneuron-derived Ntf3 is essential for survival of proprioceptive neurons in the DRG. This study implies motoneuron as a functional source of Ntf3, a pre-target neurotrophin for sensory neurons.

Effects of motoneuron deficiency on sensory neuron development

In the DRG of *Olig2* KO embryos, increased apoptosis was observed only at E10.5 but not at E13.5 (Fig. 2A-E), suggesting motoneuron-derived neurotrophin(s) have an important role at the early stage. Consistently, we observed decreased expression of *Ntf3* in motoneurons, and increased expression in the mesenchyme and target organs at E13.5. As DRG apoptosis peaks around embryonic stage E12-E14 (Sun et al., 2008), it is difficult to detect any subtle increase in apoptosis caused by the motoneuron deficiency in *Olig2*

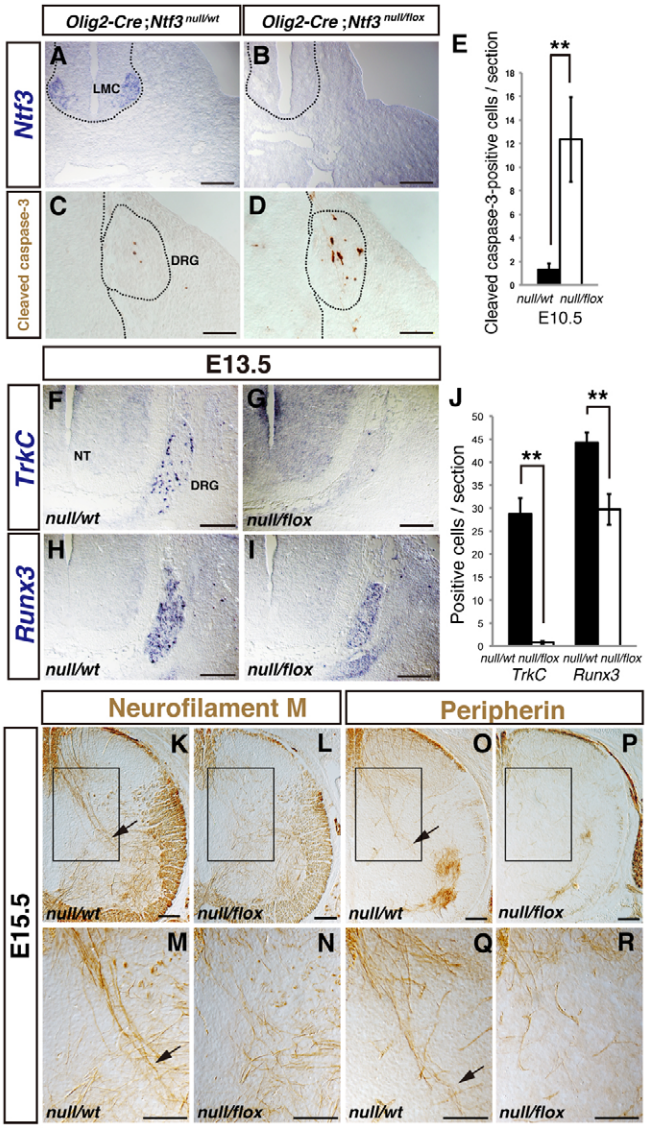

Fig. 5. Depletion of motoneuron-derived *Ntf3* caused cell death in the DRG. (**A**,**B**) DIC micrographs of *Ntf3* in situ hybridization in the spinal cord transverse sections of *Olig2-Cre*; *Ntf3*$^{null/wt}$ (A) and *Olig2-Cre*; *Ntf3*$^{null/flox}$ (B) embryos at E10.5. *Ntf3* expression was not observed in the LMC of *Ntf3* cKO mice. Dotted lines indicate margins of the spinal cord. LMC, lateral motoneuron column. (**C**,**D**) Cleaved caspase 3 immunostaining in *Olig2-Cre*; *Ntf3*$^{null/wt}$ (C) and *Olig2-Cre*; *Ntf3*$^{null/flox}$ (D) embryos at E10.5. DRG, dorsal root ganglion. (**E**) Quantitative analysis of cleaved caspase 3-positive cells in the DRG at E10.5. Values are mean±s.d. (**P<0.01, *t*-test). Dotted lines indicate margins of the spinal cord and DRG. (**F**,**G**) *TrkC* in situ hybridization in *Olig2-Cre*; *Ntf3*$^{null/wt}$ (F) and *Olig2-Cre*; *Ntf3*$^{null/flox}$ (G) embryos at E13.5. NT, neural tube. (**H**,**I**) *Runx3* in situ hybridization in *Olig2-Cre*; *Ntf3*$^{null/wt}$ (H) and *Olig2-Cre*; *Ntf3*$^{null/flox}$ (I) embryos at E13.5. The number of *TrkC*-positive and *Runx3*-positive cells was reduced in the DRG of *Olig2-Cre*; *Ntf3*$^{null/flox}$ embryos at E13.5. (**J**) Quantitative analysis of *TrkC*-positive and *Runx3*-positive cells in the DRG at E13.5. Values are mean±s.d. (**P<0.01, *t*-test). (**K-N**) Neurofilament M immunostaining in *Olig2-Cre*; *Ntf3*$^{null/wt}$ (K) and *Olig2-Cre*; *Ntf3*$^{null/flox}$ (L) embryos at E15.5. (M,N) Higher magnification views of boxed areas in K,L, respectively. (**O-R**) Peripherin immunostaining in *Olig2-Cre*; *Ntf3*$^{null/wt}$ (O) and *Olig2-Cre*; *Ntf3*$^{null/flox}$ (P) embryos at E15.5. (Q,R) Higher magnification views of boxed areas in O,P, respectively. Arrows indicate Ia afferents, which are absent in the spinal cord of *Olig2-Cre*; *Ntf3*$^{null/flox}$ embryos. Scale bars: 100 μm in A,B,K-R; 50 μm in C,D,F-I.

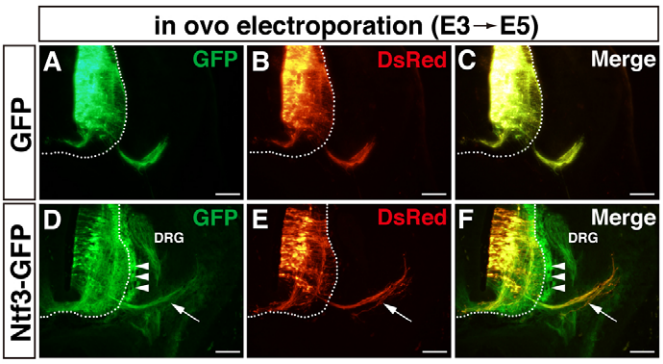

Fig. 6. Secreted Ntf3-GFP protein reaches DRG in vivo. (**A-C**) Fluorescence micrographs of transverse sections of GFP and DsRed-electroporated chick embryo at E5, GFP staining (green) (A) and intrinsic fluorescence DsRed (red) (B) were merged in C. (**D-F**) Ntf3-GFP and DsRed-electroporated chick embryo at E5, GFP staining (green) (D) and intrinsic fluorescence DsRed (red) (E) were merged (F). Arrows indicate motoneuron axons, arrowheads indicate secreted Ntf3-GFP proteins from neural tube. DRG, dorsal root ganglion. Scale bars: 100 μm.

KO embryos at E13.5. For proper axonal projection of peripheral branches, not only the secretion of soluble factors from motoneurons, but also the contact-mediated mechanism between motor axons and sensory axons (Landmesser and Honig, 1986) may play important role. Disruption of (any or both of) these mechanisms may account for the abnormal axonal projection of the peripheral branch of sensory neurons in *Olig2* KO mice. It is possible that abnormal peripheral branch projection results in abnormal central branch projection (Patel et al., 2003). In addition, because Ia afferents of proprioceptive neurons make synapses with motoneurons, such synapses cannot be formed in the Olig2 KO embryos, which have no motoneurons. It appears that multiple mechanisms may exist through which a motoneuron defect could affect the projection of sensory neurons. It was recently demonstrated that epaxial motor axon specific-EphA3/4 is essential for epaxial sensory axon to track along the preformed motor axon (Wang et al., 2011).

Role of motoneuron-derived Ntf3 on spinal circuit formation

Because of the specific expression of *Ntf3* in motoneurons at the early stage and both *Ntf3* KO mice (ElShamy and Ernfors, 1996) and *Olig2* KO mice (this study) show some comparable phenotypes, we also generated *Ntf3* cKO embryos using *Olig2-Cre* driver mice to investigate the role of motoneuron-derived Ntf3 on sensory neuron development. The number of apoptotic cells in the E10.5 DRG is higher in *Olig2* KO mice (49.7±11.4; Fig. 2) than in *Ntf3* KO mice (20.3±8.3). There are two possibilities to explain this phenomenon: first, the neurotrophic factors other than Ntf3 may have roles to prevent apoptosis; and second, the transaxonal transmission is missing in motoneuron-deficient *Olig2* KO mice.

Our data from *Ntf3* cKO embryos indicate that the motoneuron-derived Ntf3 acts as a pre-target neurotrophin to prevent apoptosis at the early stage of neural circuit formation in vivo (Fig. 7). As sensory neurons locate very close to motoneurons at this early stage (Fig. 7), it is conceivable that the motoneuron-derived Ntf3 can act as a local signal to prevent apoptosis of sensory neurons. As the number of apoptotic cells in *Ntf3* KO embryos is higher than that of *Ntf3* cKO embryos (20.3±8.3 in Ntf3 KO; 12.4±3.6 in Ntf3 cKO, Fig. 5E), Ntf3 from tissues other than motoneurons may also have some trophic functions. Another possible role of the motoneuron-derived Ntf3 at the early stage is to support outgrowth or attraction of the peripheral branch (Fig. 7), because NT-3 has role in sensory axonal projection, as shown previously by gain-of-function and loss-of-function experiments in vivo (Lefcort et al., 1996; Tucker et al., 2001). The reason why there is no obvious defect in peripheral branch in the *Ntf3* cKO mice might be attributed to the presence of many repulsive cues around the DRG (Keynes et al., 1997; Masuda et al., 2008); that is, sensory axons may finally find their route and fasciculate with motoneuron axons without attractive cues. After fasciculation, it is possible that Ntf3 is secreted from motor axons as a transaxonal signaling factor, analogous to the secretion of Sema3a and Sema3f from developing axons (Moret et al., 2007; Takeuchi et al., 2010). At E15, central branches of sensory neurons enter spinal cord from the dorsal root entry zone after the waiting period (Ozaki and Snider, 1997; Watanabe et al., 2006), and, then, proprioceptive axons directly innervate motoneurons. We observed that Ia afferents did not project towards ventral motoneurons in the *Ntf3* cKO mice (Fig. 5L,N,P,R). This is attributable to the loss of *TrkC*-positive neurons during DRG development in *Ntf3* cKO embryos (Fig. 5).

Neurotrophins including Ntf3 have multiple roles in not only developing brains but also adult brains and pathological brains. Neurotrophins are secreted in an activity-dependent manner from mature neurons to regulate synaptic plasticity (Je et al., 2005; Vicario-Abejon et al., 2002). Exogenous Ntf3 expression in target nucleus facilitates axonal regeneration and synapse formation after the spinal cord injury (Alto et al., 2009; Kadoya et al., 2009) and

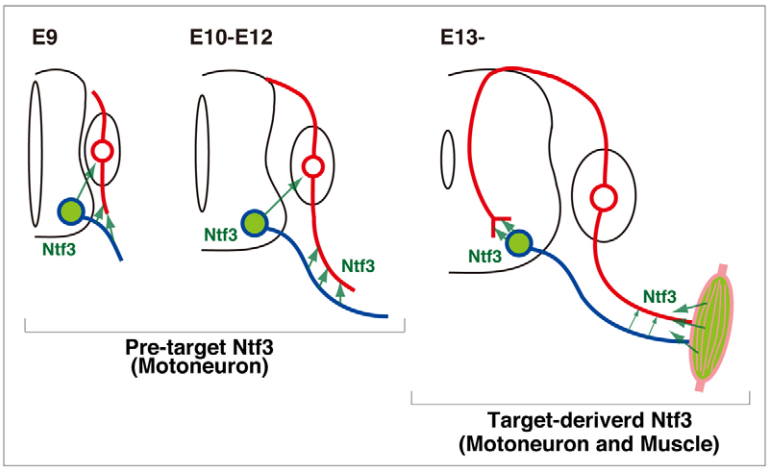

Fig. 7. A hypothetical scheme of the sensory-motor communication in the developing spinal circuit via motoneuron-derived Ntf3. At around E9, DRG locates close to the neural tube and motoneurons strongly express Ntf3. Sensory neurons receive motoneuron-derived Ntf3 as a survival factor. At E10 to E12, Ntf3 acts as a pre-target neurotrophin. After E13, the motoneuron-derived Ntf3 expression becomes weaker, whereas the target-derived Ntf3 expression becomes stronger, as in the muscle and skin. After the waiting period, sensory neurons extend their central branches into the spinal cord at E13 and Ia afferents then innervate motoneurons. At these stages, Ntf3 acts as a target-derived neurotrophin. Motoneuron, blue; sensory neuron, red; Ntf3, green; muscle, pink. Arrows indicate secreted Ntf3.

such findings indicate that Ntf3 can be a candidate factor for promoting neuronal regeneration after injury. Accordingly, understanding the mechanism of neurotrophin secretion and function during development is crucially important for the field of CNS regeneration. Our study implies the possibility of using Ntf3 as a pre-target neurotrophin to facilitate the axonal regeneration.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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