Axonal projections of mechanoreceptive dorsal root ganglion neurons depend on Ret

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SUMMARY
Establishment of connectivity between peripheral and central organs is essential for sensory processing by dorsal root ganglion (DRG) neurons. Using Ret as a marker for mechanoreceptive DRG neurons, we show that both central and peripheral projections of mechanoreceptive neurons are severely impaired in the absence of Ret. Death of DRG neurons in Ret-deficient mice can be rescued by eliminating Bax, although their projections remain disrupted. Furthermore, ectopic expression of the Ret ligand neurturin, but not Gdnf, in the spinal cord induces aberrant projection of mechanoreceptive afferents. Our results demonstrate that Ret expression in DRG neurons is crucial for the neurturin-mediated formation of precise axonal projections in the central nervous system.

KEY WORDS: Neuroscience, Axon guidance, Mechanoreceptive neuron, Sensory neuron, Mouse

INTRODUCTION
Establishing precise neuronal circuits during development is essential for the proper execution of various neural activities. Dorsal root ganglion (DRG) neurons form a circuit that relays signals from peripheral sensory organs to the central nervous system. DRG neurons that transmit different sensory modalities project their axons to distinct laminae in the spinal cord (Mirmics and Koerber, 1995b; Sanes and Yamagata, 1999), and the development of these projections is a complex process (Altman and Bayer, 2001). First, DRG proximal afferents travel to the spinal cord, where they bifurcate and extend ascending and descending branches within the prospective dorsal funiculus. Then, collaterals invade the gray matter of the spinal cord and terminate in the target lamina. Finally, the ascending fibers of DRG neurons within the dorsal funiculus proceed to the dorsal column nuclei of the medulla. Peripheral projections of sensory afferents have been examined in detail in rat embryos (Mirmics and Koerber, 1995a). First, afferent fibers exit the lumbosacral DRG at E12, and by E14 they are present in the epidermis of the proximal hindlimb. Fibers originating from L3 to L5 reach the paw by E14.5-15, and the epidermis of the most distal toes is innervated by E16-16.5. Given that the development of afferent projections in the spinal cord has been shown in several studies to be delayed relative to the innervation of the hindlimb, it has been proposed that peripheral innervation may stimulate central axon growth (Smith and Frank, 1988). However, recent experiments using carbocyanine dyes argue against this hypothesis (Mirmics and Koerber, 1995a).

DRG neurons can be divided into three groups based on sensory modality: nociceptive, mechanoreceptive and propriocceptive (Marmigere and Ernfors, 2007). Nociceptive afferent neurons penetrate into the dorsal horn of the spinal cord and terminate in laminae I and II. Mechanoreceptive neuron afferents invade the medial gray matter of the spinal cord, then turn and enter the dorsal horn ventrally to terminate in laminae III and IV of the dorsal horn. Proprioceptive afferents pass through the medial part of the dorsal horn without branching and reach the ventral spinal cord, where they finally synapse to motoneurons (Caspary and Anderson, 2003). Whereas the axon terminals of nociceptive neurons are strictly confined to the dorsal horn and do not enter the ventral spinal cord, proprioceptive central afferents do not branch in the dorsal horn and are able to invade the ventral spinal cord (Eide and Glover, 1997). Thus, it has been suggested that repulsive and attractive cues residing in the dorsal horn and/or the ventral spinal cord guide each afferent to the proper region (Perrin et al., 2001; Sharma and Frank, 1998). However, despite some progress (Frank, 2006), the mechanisms governing central afferent projections to the spinal cord remain unclear.

Neurotrophins [Ngf, Bdnf, NT-3 (Ntf3) and NT-4 (Ntf5)] play a crucial role in the differentiation, innervation pattern and survival of sensory neurons (Lewin and Barde, 1996; Markus et al., 2002). The sensory modality of a DRG neuron is tightly correlated with Trk subtype expression (Moqrich et al., 2004; Sun et al., 2008). Nociceptive neurons express TrkA (Ntrk1) and proprioceptive neurons express TrkC (Ntrk3). However, only a subset of mechanoreceptive neurons expresses TrkB (Ntrk2) (Gonzalez-Martinez et al., 2004), and no histochemical marker for all mechanoreceptive neurons has yet been identified (Snider and Wright, 1996).

Ret is a receptor tyrosine kinase that binds to the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) Gdnf, neurturin (Nrttn), artemin and persephin (Airaksinen and Saarma, 2002). Recent studies of genetically modified mice have demonstrated that GFL/Ret signaling is important for cell migration, axonal outgrowth and cell survival during peripheral nervous system development (Fundin et al., 1999; Kramer et al., 2006a). In the sensory nervous system, Ret expression first starts in TrkA+ small-diameter neurons during embryonic development, but these Ret+ neurons downregulate TrkA expression and eventually become TrkA-, nonpeptidergic, isoleucine-B4 (IB4)+ nociceptive DRG neurons after birth, while Ret+ TrkA+ neurons become peptidergic nociceptive neurons. Ret is responsible for the acquisition of several properties
of nonpeptidergic sensory neurons (Luo et al., 2007; Molliver et al., 1997). Since Ret-deficient mice die perinatally due to renal agenesis, conditional deletion of Ret in sensory neurons has been performed to analyze the role of Ret in nonpeptidergic nociceptive neuron development (Luo et al., 2007). Loss of Ret affects only the peripheral, and not the central, projections of nonpeptidergic nociceptive neurons. In addition, the number of cells expressing Gfrα1 and Gfrα2 is significantly reduced in conditional Ret-null mutants, whereas nonpeptidergic neuronal loss is not observed. Thus, Ret signaling regulates the expression of these Gfrα co-receptors during nonpeptidergic nociceptive neuron development, without affecting cell survival in these neuronal populations.

Furthermore, the expression of Ret in small TrkA+ nociceptive neurons is regulated by the neurotrophic factor Ngf, which instructs neuronal fate by suppressing TrkA expression (Luo et al., 2007). Several experiments have provided evidence that Ret is also expressed in large-diameter DRG neurons from early in DRG development (Kramer et al., 2006b; Luo et al., 2007), and Ret+ fibers have been observed in the deep dorsal horn. However, the physiological role of Ret in large-diameter DRG neurons is not well understood (Ersnberger, 2008). In order to determine whether Ret signaling plays a role in large-diameter neurons, we examined Ret mutant mice in which tau-GFP was knocked into the Ret locus. We found that Ret is required for the projections of mechanoreceptive neurons during development.

MATERIALS AND METHODS

Animals

The generation of RetGFP/GFP and Gfra3−/− mice was described previously (Enomoto et al., 2001; Honma et al., 2002). Bax-deficient mice were purchased from Jackson Laboratory (Knudson et al., 1995). Nrtn-GFP transgenic mice were obtained from Mutant Mouse Regional Resource Centers (Gong et al., 2003). This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals from the Society for Neuroscience and was authorized by the Animal Care and Use Committee of RIKEN.

In utero electroporation

The introduction of DNA into E12.5 spinal cord was performed by in utero electroporation. The expression vector (1-5 µg/µl) was mixed with 0.05% Fast Green as a tracer and injected into the central canal of the embryo through the uterine wall. After injection, electrodes (CUY 650-5, Nepa Gene) were placed on both sides of the embryo and electroporation was performed using a square-pulse electroporator CUY21 EDIT (Nepa Gene).

Antibodies

The following antibodies were used: mouse anti-Isl1 (DSHB); rabbit anti-cleaved caspase 3 and rabbit anti-Aml1 (Runx1) (Cell Signaling); rabbit anti-Runx3 (Osaki et al., 2004); goat anti-Gfrα1, goat anti-Gfrα2, goat anti-Ret, goat anti-TrkB, goat anti-TrkC and goat anti-neurturin (R&D); rabbit anti-Ret (IBL); rabbit anti-TrkA, rabbit anti-NF-200, rabbit anti-peripherin and guinea-pig anti-vGlut1 (Millipore); mouse anti-cytokeratin (Kramer et al., 2006b; Luo et al., 2007), and Ret+ expressed in large-diameter DRG neurons from early in DRG development (Kramer et al., 2006b; Luo et al., 2007), and Ret+ neurons is regulated by the neurotrophic factor Ngf, which instructs neuronal fate by suppressing TrkA expression (Luo et al., 2007). Several experiments have provided evidence that Ret is also expressed in large-diameter DRG neurons from early in DRG development (Kramer et al., 2006b; Luo et al., 2007), and Ret+ fibers have been observed in the deep dorsal horn. However, the physiological role of Ret in large-diameter DRG neurons is not well understood (Ersnberger, 2008). In order to determine whether Ret signaling plays a role in large-diameter neurons, we examined Ret mutant mice in which tau-GFP was knocked into the Ret locus. We found that Ret is required for the projections of mechanoreceptive neurons during development.

RESULTS

Two distinct types of Ret+ DRG neurons during development

To characterize Ret expression in the sensory nervous system, we used mice in which tau-GFP was knocked into the Ret locus (RetGFP/GFP) to map the projections of Ret+ DRG neurons in vivo (Enomoto et al., 2001). Consistent with previous data, the GFP reporter accurately reflected the Ret expression pattern. To assess which population of early embryonic DRG neurons expresses Ret and to examine the central and peripheral axonal projection patterns of Ret+ neurons, we examined GFP expression in an extensive array of sensory neuron targets.

DRG neurons began expressing GFP at ~E11.5 (see Fig. S1A,B in the supplementary material). Most of these GFP+ neurons had larger somata than TrkA+ small-diameter neurons. Few, if any, small-diameter DRG neurons, which express TrkA, were GFP+ even at E14.5 (see Fig. S1C in the supplementary material). However, by E18.5, the number of GFP+ small-diameter neurons had increased, even though the percentage of GFP+ large-diameter DRG neurons did not change during embryonic development (E13.5-18.5), remaining at ~7-10% of Isl1+ neurons. This suggests that there are two distinct populations of Ret+ DRG neurons (Kramer et al., 2006b). One is a small population of large-diameter neurons that begin expressing Ret at E11.5, the percentage of which, relative to the total number of DRG neurons, is stable throughout embryonic development. The other population comprises small-diameter neurons that emerge at ~E13.5, express TrkA (see Fig. S1D in the supplementary material), and gradually become the major population of Ret+ DRG neurons at later stages of embryonic development.

Development of Ret+ central afferents in the spinal cord

We carefully examined GFP+ axonal projections in the spinal cord during early embryonic stages (E12.5-16.5) in order to understand the behavior of central afferent projections of Ret+ large-diameter neurons. The axon collaterals of GFP+ DRG neurons began to penetrate the spinal cord gray matter at E12.5 (Fig. 1C). At E12.5,
Mechanoreceptive afferents terminating in the deep dorsal horn are severely disturbed in Ret-deficient mice

To determine whether loss of Ret affects the development of the mechanoreceptive sensory neurons that normally express Ret during embryonic development, we examined the phenotype of GFP+ fibers in RetGFP/GFP mice. GFP+ DRG neurons were detected in RetGFP/GFP and Ret+/GFP mice at E11.5 (see Fig. S1A,B in the supplementary material). At E12.5, the first GFP+ axonal projections were observed, but no GFP+ axon collaterals were observed in the spinal cord gray matter in RetGFP/GFP mice (Fig. 1D). At E13.5, GFP+ axon collaterals were found in the gray matter of RetGFP/GFP mice (Fig. 1G), but were few in number compared with those in Ret+/GFP mice (Fig. 1E-G). Although the number of axon collaterals invading the spinal cord increased from E13.5-14.5 in RetGFP/GFP mice (Fig. 1G), but still sparse compared with those in Ret+/GFP mice (Fig. 1F). Axon collaterals in Ret+/GFP mice project laterally (H), but those of RetGFP/GFP mice appear to lose their direction at E14.5 (I). Collaterals finally terminate in the deep dorsal horn with upward endings at E16.5 in Ret+/GFP mice (J), but these fibers are severely impaired in RetGFP/GFP mice (K). Vertically running fibers projecting to the medulla within the dorsal funiculus in Ret+/GFP mice (arrow in H) are severely impaired in RetGFP/GFP mice at E14.5 (arrow in I). (L,M) Central aff erent projections into the deep dorsal horn are apparent even at P6 (L), and these afferents are also labeled with vGlut1, a marker for the deep dorsal horn (M). (E) Central afferent projections are significantly reduced in RetGFP/GFP mice at E12.5 and E13.5. n=3 for each group. *P<0.05; **P<0.01 (Student’s t-test). Scale bars: 50 μm.

The Ret function in mechanoreceptive neurons

Mechanoreceptive afferents terminating in the deep dorsal horn are affected in Ret+/GFP mice. We first performed DiI tracing to label all centrally projecting DRG afferents. DiI labeling at E16.5 revealed severe fiber loss in the deep dorsal horn, despite a lack of obvious abnormalities in proprioceptive axonal projections to the ventral spinal cord (Fig.
found similar abnormalities at E18.5 in the deep dorsal horn of mice. (E-H) Parvalbumin+ proprioceptive afferents and TrkA+ nociceptive afferents are identical in Rett+/GFP and Rett+/GFP mice. (I-N) Double immunostaining for GFP and TrkA, vGlut1 and parvalbumin was performed at E18.5. GFP+ fibers are impaired (J,L,N) and double staining of GFP with vGlut1 is severely reduced in Rett+/GFP mice (arrowheads in I,J). Punctate GFP staining reflects Rett+ cells in the deep dorsal horn (J,L,N). Scale bars: 50 μm.

2A,B). We then investigated the expression of vGlut1 because it marks the central afferents that transmit low-threshold mechanoreceptive sensory inputs to the deep dorsal horn of the spinal cord. We found that vGlut1 expression was exclusively eliminated from laminae III-IV, but was retained in the intermediate and ventral gray matter of spinal cord at E16.5 in Ret-deficient mice (Fig. 2C,D). Thus, all mechanoreceptive sensory afferents are lost from the deep dorsal horn in the absence of Ret. Consistent with the preservation of vGlut1 expression in the intermediate and ventral spinal cord in Ret+/GFP mice, parvalbumin+ proprioceptive afferents were not affected (Fig. 2E,F). In addition, TrkA+ cutaneous nociceptive afferents in laminae I-II were comparable in Ret+/GFP and Ret+/GFP mice (Fig. 2G,H). We also found similar abnormalities at E18.5 in the deep dorsal horn of Ret+/GFP mice (Fig. 2I-N). These results indicate that Ret is exclusively required for mechanoreceptive neuron terminals in the deep dorsal horn, and that loss of these terminals does not result in invasion of proprioeptive and nociceptive afferents into the deep dorsal horn.

Since laminar structure could influence the lamina-specific termination of DRG neurons, we examined the laminar structure of the dorsal horn using lamina-specific molecular markers, including Ebf1, Drg11 (Prx1 – Mouse Genome Informatics), Brn3a (Pou4f1) and c-Maf (Li et al., 2006). At E14.5, expression of these molecules in dorsal horn was normal in Ret+/GFP mice, suggesting that the dorsal horn structure is not impaired by the lack of Ret (see Fig. S2A-H in the supplementary material), despite expression of Ret in the developing dorsal horn (see Fig. S2I in the supplementary material). Thus, it is unlikely that the defects in mechanoreceptive central afferent projections are due to aberrant dorsal horn formation.

**Ret+ fibers innervate mechanoreceptors in the periphery and are severely impaired in Ret-deficient mice**

Next, we examined GFP-labeled peripheral projections in Ret+/GFP mice. Because we still observed GFP immunostaining in the deep dorsal horn, as described above (Fig. 1L,M), it seemed likely that GFP staining marks early large-diameter Rett+ DRG sensory neuron fibers during early postnatal stages. Thus, we examined GFP+ axon terminals in the forelimb skin at P3-P6. GFP+ fibers with lanceolate or Ruffini endings were found around hair follicles (Fig. 3A). Furthermore, we found GFP+ fibers innervating several types of mechanoreceptors, including Meissner corpuscles (Fig. 3D), Merkel cells (Fig. 3E) and Pacinian corpuscles (Fig. 3F) in the skin and the crural interosseous membrane. Thus, Rett+ DRG neurons project into the deep dorsal horn (laminae III-IV) and innervate mechanoreceptors in the skin, suggesting that large-diameter Rett+ DRG neurons are mechanoreceptive neurons.

To explore whether Ret deficiency also influences peripheral projections, we examined GFP+ fibers in the body wall skin at E12.5 and in the forelimb skin at E18.5. As expected, NF-200 (Nefh – Mouse Genome Informatics) immunostaining, which marks prospective myelinated axons (Zylka et al., 2005), was significantly reduced in the skin of E18.5 Ret+/GFP mice (Fig. 3H). However, these axons were not completely lost, consistent with the observation of a few GFP+ fibers in Ret+/GFP skin (Fig. 3G). By contrast, fibers that are positive for peripherin, a marker for prospective unmyelinated axons (Kosaras et al., 2009; Lariviere et al., 2002), were unaffected in Ret+/GFP mice (Fig. 3I), consistent with the idea that Ret+ neurons are myelinated mechanoreceptive neurons, although the staining specificity of NF-200 and peripherin is somewhat controversial (Jackman and Fitzgerald, 2000). To examine the cutaneous branch of the sensory nerve in Ret-deficient mice, we performed whole-mount NF-200 and peripherin immunostaining of the dorsal skin of the left upper forelimb at E14.5. NF-200+ fibers in Ret+/GFP mice were thinner than those in Ret+/GFP mice, but their branching appeared to be intact (Fig. 3J,K). By contrast, peripherin+ fibers were not impaired in Ret+/GFP mice (Fig. 3L,M). Thus, our data show that the peripheral projections of NF-200+ sensory fibers are severely reduced in Ret+/GFP mice.
Ret function in mechanoreceptive neurons

The role ofNrtn-Gfrα2 in Ret+ mechanoreceptive neurons

GFL signaling requires a receptor complex comprising Ret and a Gfrα co-receptor. Since the effects of Ret deficiency on the expression of Gfrα co-receptors have only been examined during the later stages of embryonic development (Luo et al., 2007), we examined co-receptor expression in DRG neurons during early embryogenesis in Ret mutant mice. Because we did not observe any abnormalities in central and peripheral Ret+ DRG afferents in Ret+/GFP, Gfra3−/− mice (see Fig. S3A-D in the supplementary material), we focused on Gfrα1 and Gfrα2. Antibodies against either Gfrα1 or Gfrα2 and GFP were used to double label DRG neurons. At E13.5, Gfrα2+ cells (93±1%) were GFP+ large-diameter neurons (Fig. 4D-F), whereas Gfrα1+ cells (30±2.8%) were GFP+ small-diameter neurons (Fig. 4A-C). Thus, during embryonic development, Gfrα2 is the best candidate for a Ret co-receptor in large-diameter DRG neurons.

Because Gfrα2 is a known co-receptor for Nrtn (Heucker et al., 1999; Rossi et al., 1999), we next examined Nrtn expression during embryonic development. Since Nrtn expression is scarcely detectable by in situ hybridization, we used transgenic mice carrying GFP under the control of the Nrtn promoter in order to follow Nrtn expression during development. We found Nrtn expression in the DRG and in peripherally projecting axons at E12.5 (Fig. 4J). At E13.5, Nrtn was expressed in the dorsal root entry zone and in the path of the central afferents (Fig. 4K). We also detected signal in the prospective dorsal funiculus and deep dorsal horn at E13.5 (Fig. 4M). Although Nrtn expression in the proximal afferents and DRG became weak at E14.5 (data not shown), expression in the deep dorsal horn was clearly evident at E14.5 (Fig. 4O). The signal in the deep dorsal horn was scattered at E13.5 (Fig. 4M), but was concentrated on the lateral side of the dorsal horn at E14.5 (Fig. 4O). The Nrtn signal appeared to precede the GFP+ fiber projections in the deep dorsal horn of Ret−/− mice (Fig. 4L-O). Finally, the Nrtn expression level in the dorsal horn was decreased by E16.5 (data not shown). At E15.5, Nrtn was also expressed in the peristeum and ligaments of the developing forelimb at E12.5 (data not shown) and around the hair follicles, a potential target of mechanoreceptive neurons (Fig. 4P).

Intriguingly, we observed that Ret+ central afferents terminated in the deep dorsal horn in chick embryos (Fig. 4Q) and that Nrtn expression during development. We found Nrtn expression in the DRG and in peripherally projecting axons at E12.5 (Fig. 4I). At E13.5, Nrtn was expressed in the dorsal root entry zone and in the path of the central afferents (Fig. 4K). We also detected signal in the prospective dorsal funiculus and deep dorsal horn at E13.5 (Fig. 4M). Although Nrtn expression in the proximal afferents and DRG became weak at E14.5 (data not shown), expression in the deep dorsal horn was clearly evident at E14.5 (Fig. 4O). The signal in the deep dorsal horn was scattered at E13.5 (Fig. 4M), but was concentrated on the lateral side of the dorsal horn at E14.5 (Fig. 4O). The Nrtn signal appeared to precede the GFP+ fiber projections in the deep dorsal horn of Ret−/− mice (Fig. 4L-O). Finally, the Nrtn expression level in the dorsal horn was decreased by E16.5 (data not shown). At E15.5, Nrtn was also expressed in the peristeum and ligaments of the developing forelimb at E12.5 (data not shown) and around the hair follicles, a potential target of mechanoreceptive neurons (Fig. 4P).

Characterization of DRG neuron abnormalities in Ret-deficient mice

Several lines of evidence indicate that functionally distinct DRG neurons express specific neurotrophic factor receptors, and that this diversification underlies the segregation of DRG neurons according to sensory modality (Woolf and Ma, 2007). To assess whether the loss of Ret influences the segregation of neurotrophic factor receptor expression in DRG neurons, we performed double-labeling experiments at E13.5 with an anti-GFP antibody and antibodies against different neurotrophic factor receptors. There was little overlap between GFP expression and neurotrophic factor receptor expression in the DRG neurons of E13.5 Ret−/− mice (Fig. 5C,E,G), in agreement with a previous report (Kramer et al., 2006b). The GFP and Trk receptor expression patterns in Ret−/−/GFP+ mice were almost identical to those in Ret+/−/GFP+ mice (Fig. 5C-H), suggesting that Ret signaling is not involved in the segregation of Trk receptor expression. Furthermore, we found that there was little overlap between GFP expression and that of Runx1 (a marker for nociceptive neuron) or Runx3 and parvalbumin (markers for proprioceptive neurons) in Ret−/−/GFP+ mice (Patel et al., 2003; Sun et al., 2008; Yoshikawa et al., 2007), and the expression...
pattern in RetGFP/GFP mice was comparable to that in Ret+/GFP mice (Fig. 51-N). These observations indicate that Ret is not involved in the segregation of DRG neurons with respect to sensory modality. Because GFP+ afferent projections were impaired during early embryogenesis in Ret mutants, we examined whether Ret functions in mechanoreceptive neurons during early embryonic development. We first tested whether impaired central and peripheral DRG projections in Ret-deficient early embryos affect sensory neuron development. In situ hybridization revealed loss of Gfra2 expression in the DRG of E14.5 RetGFP/GFP mice (Fig. 6C,D), whereas Gfra1 expression was identical to that of controls (Fig. 6A,B). We then quantitatively examined the number of Isl1+, GFP+, Gfra1-expressing and Gfra2-expressing neurons (Fig. 6E). Isl1 labeling was used to evaluate the total number of DRG neurons. We detected a 37% reduction in the number of GFP+ neurons in Ret-deficient embryos at E14.5, even though the number of Isl1+ neurons was not significantly reduced. The small neuron reduction in RetGFP/GFP mice at E14.5, before the stage at which we observed the massive reduction in number, which might reflect the observation that most peripherin+ neurons are hypotrophic in Ret-deficient mice, as previously described (Luo et al., 2007). Collectively, these results indicate that large-diameter sensory neurons are severely reduced in the DRG of Ret-deficient mice.

**Bax deficiency rescues loss of GFP+ and Gfra2-expressing neurons, but fails to rescue mechanoreceptive neuron projection defects**

In order to determine whether the reduction in the number of neurons expressing GFP or Gfra2 was caused by cell loss or by transcriptional downregulation, we used an antibody against cleaved caspase 3 to measure cell death. The number of labeled neurons in DRG of Ret−/GFP and Ret−/GFP mice was counted at E13.5, before the stage at which we observed the massive reduction in GFP+ and Gfra2-expressing neurons (see above). There were more cleaved caspase 3+ cells in Ret−/GFP than in Ret−/GFP mice (121% of control) (Fig. 6F), suggesting that cell death is responsible for the reduction in Ret− DRG neurons at E14.5.

The axonal defects we observed might simply reflect DRG neuron loss in the absence of Ret. To eliminate the influence of cell death, we generated mice that were double null for Ret and Bax. In Bax knockout mice, cell death is virtually eliminated in peripheral ganglia, including the DRG (White et al., 1998). The number of GFP+ and Gfra2-expressing neurons in the DRG was normal in Ret−/GFP, Bax−/− mice (Fig. 6G), suggesting that Bax-mediated programmed cell death is involved in neuronal loss during
embryonic development of Ret-deficient DRG neurons. Remarkably, although the number of neurons was restored by knocking out Bax in RetGFP/GFP mice (Fig. 6G), the central and peripheral axonal projections of mechanoreceptive neurons were still impaired (69% and 89% reduction, respectively, as compared with Ret+/GFP; Bax–/– mice) (Fig. 6H-M). Taken together, these data suggest that cell death is not the direct cause of the axonal defects. Instead, the axonal projections themselves are dependent on Ret in mechanoreceptive neurons.

Ectopic Nrtn attracts Ret+ DRG central afferents to an aberrant region of the spinal cord

Given that Ret-ablated mechanoreceptive neurons in RetGFP/GFP mice fail to extend axons in the proper direction and to project into the deep dorsal horn, we hypothesized that the Ret signal might be involved in the attraction of mechanoreceptive afferents to the deep dorsal horn of the spinal cord. To test this hypothesis directly, we introduced an expression vector for the Gfrα2 ligand Nrtn into Ret+/GFP mouse spinal cords by in utero electroporation at E12.5, and harvested the embryos 3 days later to determine whether Ret+ fibers were attracted to exogenously expressed Nrtn. Consistent with our hypothesis, GFP+ fibers were attracted to the region of ectopic Nrtn expression (Fig. 7A-D). By contrast, neither Gdnf nor DsRed attracted GFP+ afferents (Fig. 7H; data not shown). TrkA+ nociceptive afferents and parvalbumin+ proprioceptive afferents in spinal cord did not respond to ectopic Nrtn expression (Fig. 7F,G), indicating that mechanoreceptive central fibers are the only afferents that are able to respond to Nrtn. Moreover, Nrtn had no attractive effect on central afferents in Ret-deficient mice (Fig. 7I), indicating that the attractive effect of Nrtn is Ret dependent.

DISCUSSION

Ret expression has been observed in large-diameter DRG neurons, but the sensory modality of these Ret+ large-diameter neurons was unclear (Ernsberger, 2008). Here, we show that Ret+ DRG neurons send peripheral axons to mechanoreceptors in the skin and central axon collaterals to the deep dorsal horn in the spinal cord. These results indicate that Ret+ large-diameter neurons are mechanoreceptive neurons. Moreover, we found that Ret is required for proper laminar termination of mechanoreceptive neuron central projections in the spinal cord. The peripheral projections of mechanoreceptive neurons are also severely disturbed in the absence of Ret. Finally, we show that Nrtn, a Ret ligand, is expressed in the region where Ret+ neurons send their axons, and that ectopic Nrtn attracts Ret+ fibers to an aberrant region of the spinal cord. These results demonstrate that Ret signaling is required for mechanoreceptive neurons to establish the circuit between peripheral and central organs, and also suggest that central afferent growth within the spinal cord is independently regulated from the signal for peripheral projections.

Neurotrophic factors for mechanoreceptive neurons

Neurotrophins (i.e. Bdnf, NT-3 and NT-4) play a developmental role in the survival, function and axonal projections of mechanoreceptive DRG neurons (Airaksinen et al., 1996; Cronk et al., 2002; Stucky et al., 1998). However, neurotrophins have only been implicated in the postnatal development of these neurons (Carroll et al., 1998), and no trophic factors involved in their development during embryonic stages have thus far been described. Our data show that Ret is essential for the development of mechanoreceptive neuron axonal projections during...
embryogenesis. Previous work showed that D-hair mechanoreceptors switch their neurotrophin dependence from NT-3 to NT-4 during postnatal development, even after DRG neurons are terminally differentiated (Stucky et al., 2002). It therefore appears that DRG neurons change their dependence on trophic factors several times before maturation.

It has been suggested that a subset of mechanoreceptive neurons express TrkB (Kramer et al., 2006b). Is there a correlation between Ret and TrkB in mechanoreceptive neuron development before birth? In TrkB-/- mice, central afferents terminating in the intermediate zone of the spinal cord, where myelinated mechanoreceptors innervate, are severely reduced, although the central afferents in the deep dorsal horn are intact (Silos-Santiago et al., 1997). By contrast, central afferents terminating in the intermediate zone are not impaired in RetGFP/GFP mice. Together with the fact that TrkB+ neuron number is not reduced in RetGFP/GFP mice, this suggests that TrkB-dependent mechanoreceptive neurons are distinct from Ret-dependent mechanoreceptive neurons. It is possible that mechanoreceptive neurons that project to the intermediate gray matter of the spinal cord depend on TrkB, but are not Ret-dependent for their innervation, at least before birth.
The role of Nrtn in the survival of mechanoreceptive neurons

Ret signaling is dispensable for the viability of nonpeptidergic DRG neurons in vivo (Luo et al., 2007). By contrast, mechanoreceptive neurons undergo cell death in Ret-deficient mice at E13.5. In mechanoreceptive neurons, Ret may function in the survival of Ret⁺ and Gfra2⁺ neurons, as Bax deficiency rescued the number of cells expressing GFP and Gfra2 co-receptors in Ret-deficient mice. However, we could not conclude whether or not Nrtn is the survival factor for mechanoreceptive neurons because a small number of GFP⁺ Gfra2⁺-expressing neurons survive in Ret°/°GFP mice. Moreover, a few GFP⁺ fibers are already found in the peripheral tissues and spinal cord of Ret°/°GFP mice at E12.5. These observations support the idea that once GFP⁺ neurons reach the skin, a target-derived trophic factor other than Nrtn promotes their survival. Indeed, GFLs fail to support embryonic DRG sensory neuron survival in the absence of Ngf in vitro, despite the expression of Ret and its co-receptors in DRG neurons (Baudet et al., 2000). It is likely that Ret signaling is required only for axonal projections, but not for the survival of mechanoreceptive neurons. However, it is still possible that other molecules, such as neurotrophins, induce mechanoreceptive neuron axonal growth even before birth, similar to what occurs in postnatal development. Thus, Nrtn might be involved in both mechanoreceptor neuron survival and axonal projections.

Nrtn is required for the axonal projections of Ret⁺ mechanoreceptive neurons

DRG neurons that convey different sensory modalities send axons into distinct laminae within the spinal cord, but the molecular mechanisms underlying lamina-specific projection patterns are largely unknown. Proprioceptive central afferents are disrupted in M3-null mice (Ernfors et al., 1994), and NT-3 attracts axons in DRG explant cultures in vitro (Genc et al., 2004). We found that mechanoreceptive neurons require Ret to establish their terminal projections in the deep dorsal horn. Ectopic Nrtn induces aberrant axonal extension of mechanoreceptive neurons via Ret in vivo. Nrtn is expressed in the dorsal horn, suggesting that Nrtn acts as a diffusible guidance cue for mechanoreceptive afferent neurons. Thus, we propose that a diffusible guidance cue exists for each class of DRG neuron that attracts afferents in the spinal cord. However, when ectopic Nrtn is introduced into the superficial dorsal horn by electroporation, Ret⁺ afferents fail to invade the region (our preliminary observation). This raises the possibility that repulsive cues in the superficial lamina of the dorsal horn prevent Ret⁺ fibers from invading the region. Sema3A repels Nfg-responsive axons, but has little effect on NT-3-responsive axons (Messersmith et al., 1995), suggesting that a repulsive cue might also exist for each class of DRG neuron. Mechanoreceptive afferents take a unique path into the spinal cord, which might indicate that they use their own repulsion molecules to terminate in the deep dorsal horn. It will be interesting to explore which repulsive cues act in concert with the attractive Nrtn cue to guide mechanoreceptive afferent projections and to reveal how precise neural circuits are established during DRG development. Repulsive cues for mechanoreceptive afferents remain to be identified, but histochemical Ret labeling should allow mechanoreceptive afferent behavior within the spinal cord to be traced. In the periphery, we have observed Nrtn expression along the peripheral sensory afferents and in their target tissues, but not along the ventral motor efferents. These findings suggest that Nrtn is required for the peripheral afferents to find their target organs, as is the case for the central projections. The generation of mice that ectopically express Nrtn in the spinal cord and/or the peripheral tissues might allow us to better understand the mechanism by which Nrtn regulates the projections of mechanoreceptive neurons.

Ret signaling in pathological conditions

Previous studies have shown that Gfra2 is the main co-receptor of Ret in nonpeptidergic nociceptive neurons (Luo et al., 2007). Thus, Gfra2 appears to play a major role in both nonpeptidergic nociceptive and mechanoreceptive Ret⁺ sensory neurons. In some pathological conditions, non-nociceptive stimuli evoke pain; for example, in allodynia, tactile stimuli cause pain (Sandkuhler, 2009). It is hypothesized that mechanoreceptive neurons undergo an abnormal phenotypic switch to nociceptive neurons in alldynia, and that non-nociceptive stimuli might evoke pain sensation through mechanoreceptive neurons. Although ligand-receptor binding may normally activate different downstream targets in different cell types and therefore elicit distinct actions, under pathological conditions it is possible that this distinction is lost. Thus, one intriguing hypothesis is that Ret-Gfra2 signaling is partly responsible for this phenotypic switch of mechanoreceptive neurons to nociceptive neurons in alldynia.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


