Molecular Identification of Rapidly Adapting Mechanoreceptors and Their Developmental Dependence on Ret Signaling

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SUMMARY

In mammals, the first step in the perception of form and texture is the activation of trigeminal or dorsal root ganglion (DRG) mechanosensory neurons, which are classified as either rapidly (RA) or slowly adapting (SA) according to their rates of adaptation to sustained stimuli. The molecular identities and mechanisms of development of RA and SA mechanoreceptors are largely unknown. We found that the “early Ret+” DRG neurons are RA mechanoreceptors, which form Meissner corpuscles, Pacinian corpuscles, and longitudinal lanceolate endings. The central projections of these RA mechanoreceptors innervate layers III through V of the spinal cord and terminate within discrete subdomains of the dorsal column nuclei. Moreover, mice lacking Ret signaling components are devoid of Pacinian corpuscles and exhibit a dramatic disruption of RA mechanoreceptor projections to both the spinal cord and medulla. Thus, the early Ret+ neurons are RA mechanoreceptors and Ret signaling is required for the assembly of neural circuits underlying touch perception.

INTRODUCTION

The perception of form and texture is fundamental and essential for the daily lives of most, if not all, animals. The first step in the perception of discriminative touch in mammals is the detection of pressure, vibration, or stretch of the skin and deflection of hairs by specialized mechanosensory end organs in the skin (Zelena, 1994). Low threshold, large-diameter trigeminal and dorsal root ganglion (DRG) neurons (mechanoreceptors) innervate these end organs and are the primary sensory neurons mediating discriminative touch and tactile perception.

DRG mechanoreceptors can be classified according to the morphologies of their peripheral end organs, which include Merkel discs, Ruffini corpuscles, Meissner corpuscles, Pacinian corpuscles, and longitudinal lanceolate endings (Albrecht and Rice, 2008; Iggo and Andres, 1982). Glabrous skin contains Merkel discs and Meissner corpuscles, whereas general hairy skin contains Merkel discs and longitudinal lanceolate endings associated with guard hair follicles. Pacinian corpuscles are found in the dermis of humans, although in mice and rats they are normally restricted to joints and the periostium of bones (Zelena, 1994). Mechanoreceptors are further distinguished as being either rapidly adapting (RA) or slowly adapting (SA) based on their rates of adaptation to sustained mechanical stimuli (Mountcastle, 1957). Meissner corpuscles, Pacinian corpuscles, and longitudinal lanceolate endings are RA mechanoreceptors (Iggo and Ogawa, 1977), whereas Merkel discs are the principle SA mechanoreceptors in rodents and monkeys (Iggo and Muir, 1969; Pare et al., 2002).

Despite physiological and morphological characterization of mechanoreceptor subtypes, mechanisms of development and unique functions of RA and SA mechanoreceptors are poorly understood, in part due to a lack of molecular identification of these neurons. Therefore, we have sought identification of candidate DRG mechanoreceptor subtypes based on a few broad criteria. First, since all mechanoreceptors are physiologically defined “Aβ” fibers, then they are almost certainly large-diameter, NF200+ DRG neurons (Lawson et al., 1993). Also, mechanoreceptors, like proprioceptors, are born shortly after coalescence of rudimentary ganglia (Lawson et al., 1974). Furthermore, mechanoreceptors account for only a small percentage of all DRG neurons (Lawson et al., 1993). Therefore, candidate mechanoreceptors must be few in number, born shortly after DRG coalescence, and have large-diameter soma sizes.

One approach to identify subtypes of DRG sensory neurons is to characterize them based on their expression of receptors for neurotrophic factors. In fact, most if not all DRG neurons express receptors for one or more neurotrophic growth factors (Marmigere and Ernfors, 2007), which promote neuronal differentiation, maturation, and survival. For example, small-diameter, unmyelinated peptidergic nociceptors express the nerve growth factor receptor TrkA and are dependent...
Figure 1. The Early Ret+ DRG Neurons Express Gfra2

(A–I) Double fluorescent in situ hybridization of Ret and Gfra1 (A–C), Ret and Gfra2 (D–F), and Ret and Gfra3 (G–I) at E13.5 (n = 3; six to eight sections were examined for each animal, lower lumbar DRGs).
on nerve growth factor-TrkA signaling for expression of nociceptor-specific genes, innervation of the epidermis, and survival (Crowley et al., 1994; Luo et al., 2007; Patel et al., 2000; Smeyne et al., 1994). Similarly, neurotrophins are involved in mechanoreceptor development and function. For example, the number of Merkel cells and their associated nerve terminals are decreased in P14 Ntr3 mutant mice (Airaksinen et al., 1996), and brain-derived neurotrophic factor (BDNF) is required postnatally for the normal transduction properties of SA mechanoreceptors (Carroll et al., 1998). In addition, overexpression of BDNF in the skin leads to enhanced innervation of hair follicles, large Meissner corpuscles, and an increase in the number of Merkel cells (LeMaster et al., 1999), whereas, conversely, Meissner corpuscles are absent in both Bdnf and TrkB null mice (Gonzalez-Martinez et al., 2004, 2005; Perez-Pinera et al., 2008). However, Pacinian corpuscles and longitudinal lanceolate endings are present in normal numbers in all neurotrophin and Trk receptor mutant mice. Thus, the identity of trophic factors and their cognate receptors that support development of most populations of RA mechanoreceptors and their peripheral and central axonal projections is lacking.

The glia-derived neurotrophic factor (GDNF) family of ligands (GFLs) contains four members, GDNF, neurturin (NRTN), artemin, and persephin (Airaksinen and Saarma, 2002). The receptors for GFLs are comprised of a signaling subunit, the receptor tyrosine kinase Ret (Durbec et al., 1996; Trupp et al., 1996), and a G-protein-coupled receptor subunit GFR alpha (Airaksinen et al., 1999). There are four GFRs in vertebrates, designated GFRa1 through GFRa4. In vitro binding assays indicate that GDNF binds preferentially to GFRa1, NRTN to GFRa2, artemin to GFRa3, and persephin to GFRa4 (Airaksinen et al., 1999). GFrα1, GFrα2, and GFrα3, but not functional GFrα4 (Lindahl et al., 2000), are expressed in unique patterns in the DRG during development (Luo et al., 2007), suggesting key roles of GFL-GFR/Ret signaling in development of distinct populations of DRG sensory neurons.

Ret is expressed in approximately 60% of adult mouse DRG neurons, which can be broadly divided into two main groups based on their development history. Most Ret+ DRG neurons are small- to medium-diameter non-peptidergic nociceptors and are born around embryonic day (E) 11.5 or later and subsequently express TrkA. Ret is not highly expressed in these neurons until E15.5 or beyond (Luo et al., 2007; Molliver et al., 1997), and recent work has revealed a central role for Ret in their maturation and epidermal innervation (Luo et al., 2007). A distinct, smaller population consists of Ret+ DRG neurons that are born earlier, express Ret prior to E11.5, do not express TrkA, and have large soma diameters. These large diameter Ret+/TrkA− neurons, which are referred to as the early Ret+ neurons (Chen et al., 2006; Kramer et al., 2006; Luo et al., 2007; Marmigere and Ernfors, 2007; Molliver et al., 1997), caught our attention because they exhibit aforementioned features of mechanosensory neurons. Here, we show that the early Ret+ DRG neurons are RA mechanoreceptors, which exhibit a modality-specific pattern of innervation of the brainstem and require Ret signaling for their assembly into neural circuits underlying discriminative touch perception.

RESULTS

Molecular Characterization of the Early Ret+ DRG Neurons

We sought molecular identification and characterization of candidate mechanoreceptors, which, like proprioceptors, are predicted to be early-born, large-diameter, NF200+ DRG neurons. One such neuronal population, the “early Ret+” neurons, uniquely fits this profile. The early Ret+ neurons are born during the first wave of DRG neurogenesis and begin to express Ret on or before E10.5, which is prior to initiation of expression of TrkA in the DRG (see Figures S1A–S1C, available online). Large-diameter, TrkC+ DRG neurons, some of which become proprioceptors (Klein et al., 1994), are born at approximately the same time. Interestingly, the early Ret+ and TrkC+ DRG neurons are distinct populations (Figures S1G–S1I; Kramer et al., 2006). Moreover, while Ret is expressed exclusively in the early Ret+ neurons (Ret+/TrkA−) in the DRG at E12 (Luo et al., 2007), it is expressed in many TrkA+ DRG neurons (Ret+/TrkA+) beginning at E13.5 (Figures S1D–S1F). Furthermore, 54.3% ± 13.4% of early Ret+ neurons coexpress TrkB at E13.5 (Figures S1J–S1L), while only 25.1 ± 7.4% of the TrkB+ DRG neurons coexpress Ret (Figures S1J–S1L), indicating that TrkB is expressed in both early Ret+ neurons as well as other DRG neurons at this time.

To characterize the early Ret+ DRG neurons with respect to their expression of Gfrα coreceptors, double fluoroscence in situ hybridization experiments were performed using cRNA probes for Ret and Gfrαs and sections from wild-type and TrkA null mice at different developmental stages. We found that Gfrα2 and Gfrα3 but not Gfrα1 are expressed in DRG neurons at E12 (Luo et al., 2007). At E13.5, Gfrα1−3 are expressed (Figures 1A, 1D, and 1G); Gfrα1 is coexpressed with Ret in many neurons (Ret+ neurons: 17 ± 4; Gfrα1+ neurons: 17 ± 5; and Gfrα1+/Ret+ neurons: 11 ± 4 neurons/DRG section; Figures 1A–1C), whereas all Gfrα2+ neurons are Ret+ (Ret+ neurons: 18 ± 2, Gfrα2+ neurons: 9 ± 2, and Gfrα2+/Ret+ neurons: 9 ± 2; Figures 1D–1F). In contrast, Gfrα3 is mainly expressed in Ret− DRG neurons at this time (Ret− neurons: 21 ± 6, Gfrα3− neurons: 51 ± 9, and Gfrα3+/Ret− neurons: 4 ± 1; Figures 1G–1I). Therefore, we focused on Gfrα1 and Gfrα2 by analyzing their patterns of expression in TrkA null DRGs. TrkA null mice were used here because the expression of Ret and Gfrα in late-born Ret+/TrkA+ DRG neurons is deficient in mice lacking TrkA.
Genetic Labeling of the Early Ret+ DRG Neurons

To test the idea that the early Ret+ neurons are mechanoreceptors, a genetic labeling approach was developed to visualize their central and peripheral axonal projections. Here, we took advantage of the observation that the early Ret+ neurons are the only DRG neurons that express Ret prior to E12.5. We generated a Ret-CreERT2 (RetERT2) knockin mouse line (Figure S3) in which myristoylated green fluorescent protein (mGFP) is expressed following Cre-mediated recombination, to generate RetERT2;Tauf(mGFP) mice. We predicted that administration of the estrogen agonist 4-HT to RetERT2;Tauf(mGFP) mice prior to E12.5 would lead to expression of GFP exclusively in early Ret+ DRG neurons (Figure 2A). In contrast, administration of 4-HT to RetERT2;TauflmGFP mice at E15.5 or later (Figure S4A) is predicted to lead to GFP expression in both the early Ret+ neurons and late-born Ret+ DRG neurons, which emerge from TrkA-precursors. Using this strategy, administration of 4-HT from E10.5 to E12.5 resulted in 280 ± 25 GFP-labeled neurons in L5 DRGs at P14 (Figure 2B), which represents ~5% of all L5 DRG neurons at this time (Liu et al., 2007). To address labeling specificity, we costained GFP+ neurons with antibodies against TrkA and Ret at both E15.5 and P0. The early Ret+ neurons should be TrkA+ and Ret+ at both ages. At E15.5, 92.5% of GFP+ neurons were TrkA+, whereas 86% of them were Ret+ (Figures 2C–2E). Similar findings were obtained with P0 pups (Figures 2F–2H). At P14, 100% of GFP+ neurons were Ret+, suggesting that the few GFP+/Ret– neurons observed at E15.5 and P0 are either lost postnatally or express Ret dynamically. In contrast, 73.7% of GFP-labeled neurons in RetERT2;TauflmGFP mice that received 4-HT at later ages (E15.5–E17.5) are TrkA+ at P0 (Figure S4), indicating that late-4-HT treatment indeed labels both the early Ret+ neurons and late-born populations of Ret+ neurons. We estimate that greater than 60% of early Ret+ neurons are labeled following administration of 4-HT to RetERT2;TauflmGFP mice from E10.5 to E12.5 by comparing the number of GFP+/TrkA– neurons (5 ± 1 neurons/DRG section; Figures 2F and 2G) to that of Ret+/TrkA– neurons at P0 (8 ± 3 neurons/DRG (Luo et al., 2007)).

To further characterize the population of Ret+ neurons labeled by administration of 4-HT from E10.5 to E12.5, we measured their soma sizes and examined their patterns of expression of GFRα2, NF200, CGRP, Parvalbumin, and TrkB and binding to the lectin IB4. As expected, the GFP-labeled early Ret+ DRG neurons have large soma sizes (average area: 587 ± 102 μm²; Figure S5A), and 72.9% ± 11% of GFP+ neurons are TrkA+ at P0 (Figures S5B–S5D). In addition, at P14, 100% of the GFP+ neurons are GFRα2+ and NF200+ (Figures 2F, 2H, and 2J). In contrast, almost none of the GFP+ neurons were found to express CGRP nor did they bind to IB4 (Figures 2J and S5H), which are markers for peptidergic (Hokfelt, 1991) and non-peptidergic nociceptors (Lawson, 1992), respectively. Furthermore, GFP+ neurons do not express Parvalbumin (Figures S5E–S5G), which labels proprioceptors (Carr et al., 1989). Thus, 4-HT treatment of RetERT2;TauflmGFP mice prior to E12.5 specifically labels the early Ret+ neurons, and these large-diameter, GFP+ DRG neurons exhibit molecular features of mechano-sensory neurons.

Figure 2. Genetic Labeling of the Early Ret+ DRG Neurons

(A) Outline of the chemical-genetic strategy for labeling the early Ret+ neurons. RetERT2 and TauflmGFP mice were crossed and timed pregnant mothers were gavaged with 1.0 to 1.5 mg 4-HT per day from E10.5 to E12.5. (B) Whole-mount anti-GFP immunostaining of a labeled L5 DRG. 280 ± 25 GFP+ neurons were labeled. Eight L5 DRGs from four animals of two litters were examined.

(C and D) Double immunostaining of GFP with TrkA (C) or Ret (D) in E15.5 labeled DRGs. 86/93 (GFP+/TrkA+ neurons/total GFP+ neurons) GFP+ neurons are TrkA+ and 233/271 (GFP+/Ret+ neurons/total GFP+ neurons) GFP+ neurons are Ret+. Lumbar DRGs; n = 6 from three litters. Four to six sections from each animal were quantified and shown in (E).

(F and G) Double immunolabelling of GFP with TrkA (F) or Ret (G) in P0 labeled DRGs. 207/225 (92%) GFP+ neurons are TrkA+ and 104/121 (86%) GFP+ neurons are Ret+. Lumbar DRGs; n = 7 from four litters for GFP/TrkA staining and n = 4 from two litters for GFP/Ret staining. Quantifications are shown in (H).

(I–L) Double immunostaining of GFP and Ret (I), GFP and GFRα2 (J), GFP and NF200 (K), and GFP, CGRP, and IB4 (L) in P14 labeled DRGs. All GFP+ neurons (95/95) are Ret+ at this time (note that, due to the fixation conditions needed for this experiment, Ret immunoreactivity is detected only in those DRG neurons expressing a high level of Ret protein). In addition, GFP+ neurons are GFRα2+ (76/76) and NF200+ (110/110), but not CGRP+ (1/82) or IB4+ (0/82). Quantifications are shown in (M). Lumbar DRGs; n = 4 from two litters. Scale bars: B, 50 μm; other panels, 20 μm.
The Early Ret+ Neurons Innervate RA Mechanosensory End Organs

Next, we examined the relationship between GFP+ fibers and the different mechanosensory end organs at P14, an age when they exhibit mature morphologies (Zelena, 1994). We first examined the innervation of Merkel discs using a TRPV3 antibody, which specifically labels Merkel cells (Figures S6A–S6F). We found that GFP+ fibers are not associated with Merkel cells in the footpad (Figures 3A–3C), touch domes of back hairy skin, guard hair follicles, or regular glabrous skin (Figures S6G–S6O). Thus, the early Ret+ neurons do not innervate Merkel cells and, therefore, they are not SA mechanosensory neurons. Instead, GFP+ fibers innervate Meissner corpuscles (Figures 3D–3F), which are located in the dermal papillae of the footpad. In hairy skin, GFP+ fibers form longitudinal lanceolate endings associated with guard hair follicles (Figures 3G–3I). Furthermore, GFP+ fibers innervate Pacinian corpuscles, which in mice are found in abundance in the periosteum of the fibula (Zelena, 1976)(Figures 3J–3L). Taken together, these findings suggest that the early Ret+ DRG neurons are RA mechanosensory neurons.
5A–5C and Figure S7) while the ventral-caudal region of the cuneate nucleus is virtually devoid of GFP+ endings (Figures 5D–5I and Figure S7). These findings reveal specific termination zones of the early Ret+ DRG neurons within the DCN of the mouse brainstem (Figure 5J), the pattern of which is remarkably similar to those of RA mechanoreceptors revealed by physiological recordings in cats (Dykes et al., 1982). Based on the peripheral and central patterns of GFP+ axonal projections, we conclude that the early Ret+ DRG neurons are RA mechanoreceptors.

Peripheral and Central Projections of Early Ret+ DRG Neurons in Retf(CFP); Wnt1Cre Mice

To further characterize the pattern of innervation of mechanosensory end organs by Ret+ DRG neurons and to substantiate our findings using RetERT2;Tauf(mGFP) reporter mice, described above, we made use of a Ret conditional CFP (Retf(CFP)) mouse line in which CFP is expressed in all Ret+ cells following CRE-mediated recombination (Uesaka et al., 2008). For these experiments, Retf(CFP) mice were crossed to a neural crest lineage mouse line, Wnt1Cre mice (Danielian et al., 1998). Thus, in Retf(CFP); Wnt1Cre mice, CFP is expressed in all Ret+ DRG neurons, including the early Ret+ DRG neurons. Consistent with our findings described above, CFP+ fibers do not innervate Merkel cells in glabrous skin or those associated with guard hair follicles in hairy skin (Figures S8A–S8C; data not shown). Instead, CFP+ fibers form Meissner corpuscles (Figures S8D–S8F), longitudinal lanceolate endings (Figures S8G–S8I), and Pacinian corpuscles (Figures S8J–S8L). Interestingly, although Wnt1Cre is active in DRG neurons, Schwann cells, and their precursors, only axons of DRG neurons are labeled with CFP (Figures S8F and S8L), indicating that Ret is expressed in RA mechanosensory DRG neurons but not Schwann cells or other neural crest-derived accessory cells. Furthermore, as expected, CFP+ fibers innervate layers I through V in the spinal cord (Figures S8M–S8P) and are highly enriched in the gracile fasciculus at the cervical level of the spinal cord (data not shown). These peripheral and central patterns of GFP+ axonal innervation indicate that a subpopulation of Ret+ DRG neurons are RA mechanoreceptors, supporting the aforementioned analysis of the early Ret+ DRG neurons using RetERT2;Tauf(mGFP) reporter mice. Hereafter, we refer to the early Ret+ DRG neurons as RA mechanoreceptors.
RA Mechanosensory Neurons Lacking Ret Signaling Components Are Present in Normal Numbers in Neonatal Mice

The high levels of embryonic expression of Ret and GFRα2, the preferred coreceptor for NRTN (Jing et al., 1997), in RA mechanosensory neurons suggests a role for the NRTN-GFRα2/Ret signaling module in controlling development of these neurons. To test this idea, we first counted the number of RA mechanoreceptors in Nrtn null (Heuckeroth et al., 1999) and Gfrα2GFP knockin mice (McDonagh et al., 2007). We measured two parameters to evaluate the number of RA mechanoreceptors at P0: the number of Ret+/TrkA− neurons in Nrtn null mice and the number of large-diameter neurons expressing a high level of GFP in Gfrα2GFP knockin mice. In both cases, similar numbers of RA mechanosensory neurons were observed in wild-type and mutant animals (Ret+/TrkA− neuron number in control and Nrtn null mice: 10 ± 1 versus 9 ± 1; GFP+ neuron number in Gfrα2GFP heterozygous versus homozygous mutant mice: 5 ± 1 versus 4 ± 1) (Figures S8A–S8H). In addition, we counted TrkB+ DRG neuron number in P0 control (21 ± 3/DRG section), Ret (21 ± 3/DRG section), Nrtn (21 ± 5/DRG section), and Gfrα2 (20 ± 4/DRG section) null mice (Figures S10A–S10D) and found virtually identical numbers of TrkB+ neurons in these mice, suggesting RA mechanoreceptors are present at P0 in the absence of Ret signaling. Consistent with these findings, our previous work revealed that normal numbers of large-diameter NF200+ neurons are found at P14 in Retf/f;Wnt1Cre mice (Luo et al., 2007). Taken together with the finding of normal numbers of cutaneous RA end organs in Ret mutants at P14, described below, we conclude that NRTN-GFRα2/Ret signaling is not required for survival of most if not all RA mechanoreceptors, at least up to P14. Interestingly, though not required for survival, Ret and Nrtn are required for maximal expression of Gfrα2 in RA mechanoreceptors as the level of Gfrα2 is diminished in both Retf/f;Wnt1Cre and Nrtn null DRGs at P0 (Figures S9A–S9C).

Since Ret signaling is not required for survival of RA mechanoreceptors and more than half of the early Ret+ neurons express TrkB at E13.5 and P0 (Figures S1L and S5K), we next asked whether TrkB controls their survival. We found a similar number of Ret+/TrkA− DRG neurons in control and TrkB+/Wnt1Cre DRGs at P0 (6 ± 2 versus 5 ± 2; Figures S10E–S10J), indicating that TrkB, like Ret, is not required for prenatal survival of most if not all RA mechanoreceptors.

Figure 5. Modality-Specific Segregation of Mechanosensory Axons in DCN
(A, D, and G) GFP+ fibers innervating the gracile and cuneate nuclei at different rostral to caudal levels of the medulla.
(B, E, and H) The VGLUT1 staining is used to visualize the gracile and cuneate nuclei.
(C, F, and I) The white dotted line outlines the boundary of the gracile nucleus (Gn) and the yellow dotted line outlines the boundary of the cuneate fasiculus and nucleus (Cn). Note that GFP+ fibers occupy most of the gracile nucleus (I), but are absent from a dorsal segment at the mid-level of the medulla (F). In contrast, GFP+ fibers do not innervate the caudal-ventral region of the cuneate nucleus (F and I), but project to the dorsal-rostral region of the cuneate nucleus (C). Arrows indicate the boundaries of the gracile and cuneate nuclei.
(j) Three-dimensional illustration of the pattern of innervation of the gracile and cuneate nuclei by RA mechanoreceptors. This model is based on the findings reported in (A–I) and in Figure S7. The VGLUT1+ zones occupied by GFP+ fibers are shown in yellow. The green zones are unoccupied VGLUT1+ zones. n = 3 for 2 month old RetERT2;Tauf(mGFP) mice treated with 4-HT from E10.5 to E12.5.
Peripheral endings of RA mechanosensory neurons were next examined for their developmental dependence on Ret signaling. Meissner corpuscles and lanceolate endings are present in normal numbers in both Retf/f;Wnt1Cre and Nrtn mutants at P14 (Figures 6C, 6D, 6G, and 6H, Figure S11C, and data not shown) although many of these endings appear morphologically underdeveloped (Figures S11A and S11B). Since Meissner corpuscles and lanceolate endings constitute the majority of RA mechanoreceptors (Johansson and Vallbo, 1979), this result supports our conclusion that Ret is not required for survival of most RA mechanosensory neurons. In contrast, mice lacking TrkB in all derivatives of the neural crest (TrkBf/f;Wnt1Cre) (Figures S11D-S11I) or TrkB null mice (Fundin et al., 1997; Gonzalez-Martinez et al., 2004, 2005; Perez-Pinera et al., 2008) exhibit a complete absence of Meissner corpuscles and a modest morphological disorganization of lanceolate endings. Interestingly, NF200+ fibers are present in the dermal papillae of the TrkBf/f;Wnt1Cre mice (Figures S11G and S11H), suggesting that mechanosensory axon terminals reach the dermal papillae but that morphological differentiation of the corpuscle fails in the absence of TrkB signaling.

In striking contrast, we found that the development of Pacinian corpuscles is absolutely dependent on Ret. These mechanosensory end organs are completely absent in the periosteum of Retf/f;Wnt1Cre mice (Figures 6A, 6B, 6E, 6F, 6I, and 6J; 35 ± 5 versus 0 Pacinian corpuscles/periosteum membrane) as determined by both S100 immunostaining and hematoxylin and eosin staining of Pacinian corpuscles in P14 control (black arrowheads, l), Retf/f;Wnt1Cre (J), Nrtn null (K), and Gfra2GFP null (L) mice. H&E staining is used here to rule out the potential confounding issue of decreased S100 expression in mutant mice. n ≥ 3 for each mutant genotype.
RA Mechanoreceptors and Ret Signaling

Since Ret is highly expressed in RA mechanosensory neurons when their axons extend into the spinal cord and then into the DCN within the medulla, we next addressed the possibility that Ret signaling controls this process. We first used VGLUT1 staining to visualize the synapses of central projections of all mechanoreceptors in RetWT;WntfCre mice and their littermate controls. Here, the intensity of VGLUT1 staining within layers III through V of the spinal cord was found to be markedly reduced in RetKO; WntfCre mice (Figures 7A, 7C, and 7D). This deficit is specific for mechanosensory endings because the innervation of both Clarke’s nucleus by proprioceptors (Figures 7A, 7C, and 7D) and layer II by non-peptidergic nociceptors (Luo et al., 2007) of the mutants is comparable to controls. In addition, the gracile nucleus is markedly smaller in Retf/f; WntfCre mice (Figures 7B, 7E, and 7F) compared to littermate controls. Furthermore, the VGLUT1 staining intensity within the gracile nucleus is dramatically reduced in Retf/f; WntfCre mice (Figures 7B, 7E, and 7G). In contrast, both the size and VGLUT1 staining intensity of the ventral-caudal region of the cuneate nucleus in Retf/f; WntfCre mice are similar to controls (Figures 7B, 7E, 7F, and 7G), which is consistent with our finding that RA mechanosensory terminations are restricted to the more medial-rostral region of the cuneate nucleus. Interestingly, TrkBWT; WntfCre mice exhibit a normal pattern of VGLUT1 staining in both the spinal cord and medulla (Figures S11K, S11L, S11N, and S11O). These results indicate that Ret, but not TrkB, is required for the formation of both branches of the central projections of RA mechanoreceptors.

Since VGLUT1 labels synapses of both RA and SA mechanoreceptors, it is possible that the central projection phenotypes observed in Retf/f; WntfCre mice reflect deficits of axonal projections of one or both of these mechanosensory populations. In addition, it is possible that Ret functions in a non-cell-autonomous manner to affect RA mechanosensory projections to the spinal cord. Indeed, Ret is expressed in other populations of DRG neurons beginning at E13.5 as well as neurons in the dorsal
spinal cord and medulla (Golden et al., 1999), all of which are subjected to Cre-mediated excision in RetERT2;Wnt1Cre mice (data not shown). To distinguish between these possibilities and address the cell autonomy of Ret function, we sought to delete Ret in RA mechanosensory neurons but not other DRG or dorsal horn neurons. Here, we used RetERT2 mice and the procedure of 4-HT treatment of mice prior to E12.5. Activation of Cre recombinase in spinal interneurons using this strategy is predicted to be minimal because expression of Ret and hence RetERT2 is seen in very few neurons in the dorsal spinal cord at E13.5 (data not shown). Indeed, we found that few spinal cord interneurons are labeled in RetERT2;TaumGFP mice following 3 days of 4-HT treatment prior to E12.5 (Figures 4A and 4E). Therefore, we generated both RetERT2;TaumGFP and RetERT2(CFP) conditional mutant mice, which enable selective visualization of the central projections of RA mechanosensory neurons that are either heterozygous or homozygous null for Ret, respectively. The RetERT2(CFP) mice harbor one RetERT2 allele, which is null, and one floxed Ret allele (ICFP), which following Cre-mediated excision is also null for Ret and expresses CFP (Uesaka et al., 2008). Thus, following 4-HT treatment at E11.5–E12.5, CFP+ DRG neurons in RetERT2(CFP) mice are Ret null RA mechanosensory neurons, whereas GFP+ neurons in control RetERT2;TaumGFP mice are RA mechanosensory neurons harboring one functional Ret allele. We used 2 days instead of 3 days of 4-HT treatment for these experiments so that relatively few RA mechanosensory neurons would be labeled. The 4-HT-treated RetERT2;TaumGFP control mice serve as an excellent control for 4-HT-treated RetERT2(CFP) mutant mice because of the following reasons. (1) The level of expression of CFP in RetERT2(CFP) mutant DRG neurons is comparable to or higher than that of GFP in control RetERT2;TaumGFP neurons, since CFP is directly visible in RetERT2(CFP) mice following recombinase, whereas GFP in TaumGFP mice is not (data not shown). (2) Two days of 4-HT treatment of RetERT2(CFP) and control RetERT2;TaumGFP mice resulted in 6 ± 2 GFP+ and 8 ± 3 GFP+ neurons/DRG section at P14 (Figures 7R and 7V), respectively, indicating the labeling efficiency in the two mouse lines is comparable. (3) Both GFP and CFP effectively localize to the central axes of mechanoreceptors, innervating layers III through V of the spinal cord of control mice (Figures 2A and 2E and Figure S8M). As predicted, 4-HT treatment at E11.5–E12.5 of both control RetERT2;TaumGFP and mutant RetERT2(CFP) mice results in very few GFP+ and CFP+ spinal cord dorsal horn interneurons, respectively (Figures 7H and 7L), indicating that Ret is deleted in very few spinal cord interneurons in RetERT2(CFP) mice. Therefore, any phenotype observed in RA mechanosensory neurons in RetERT2(CFP) mice reflects a cell autonomous function of Ret in these neurons. Using this strategy, we observed that while GFP+ neurons of RetERT2;TaumGFP control mice robustly innervate lamina III through V of the spinal cord, CFP+, Ret null neurons of RetERT2(CFP) mice exhibit a striking absence of axonal projections in both the spinal cord dorsal horn (Figures 7H, 7L, 7K, 7I, and 7M–7O) of P14 mice. Moreover, there are few GFP+ projections in the gracile and cuneate nuclei of RetERT2(CFP) mutant mice (Figures 7P, 7Q, and 7S–7U). Therefore, Ret is required cell autonomously for the extension of axonal branches of RA mechanosensory neurons into the spinal cord and the gracile and cuneate nuclei of the medulla.

To gain further insight into the nature of the central projection deficit of Ret null RA mechanoreceptors, we analyzed the axons of RetERT2(CFP) and RetERT2;TaumGFP mice at E15.5, just following the time when axons of mechanosensory neurons have sent branches into layers III through V of the spinal cord (Figures 8A and 8B; Ozaki and Snider, 1997). Mechanoreceptors typically extend a single central branch (step 1) at ~E10.5, which bifurcates upon reaching the spinal cord, projecting in both the rostral and caudal directions (step 2). Interstitial branches then form from these longitudinal projections and penetrate deep into the spinal cord (step 3) beginning at ~E13.5. Then, axonal branches in the spinal cord elaborate complicated collaterals within the proper layers of the dorsal horn (step 4) beginning at ~E15.5 (Figure 8C). To visualize individual axons of control and Ret null RA mechanoreceptors, a low dose of 4-HT was used to label 6 ± 2 GFP+ neurons/DRG in control RetERT2;TaumGFP mice and 11 ± 4 CFP+ neurons/DRG in experimental RetERT2(CFP) mice (Figures 8D–8F). We found that the initial central and peripheral axonal branches of labeled fibers within the DRGs are comparable in RetERT2;TaumGFP control (Figure 8D) and RetERT2(CFP) mutant mice (Figure 8E). Moreover, many labeled axons projecting rostrally and caudally in the dorsal funiculus, observed using sagittal sections of the thoracic spinal cord, were present in mice of both genotypes (Figures 8G and 8H). These findings indicate that axonal extension and branching steps 1 and 2 occur normally in Ret null RA mechanoreceptors. However, very few third-order axonal branches emanating from the longitudinal projections of Ret null RA mechanoreceptors were observed in RetERT2(CFP) mutant mice (0.19 ± 0.05 third-order branches/200 μm rostral-caudal fiber), which is in marked contrast to the large number of these interstitial branches seen in RetERT2;TaumGFP controls (1.48 ± 0.41 third-order branches/200 μm rostral-caudal fiber; Figures 8G–8I). This phenotype is also observed when the afferent branches are visualized using horizontal sections through the lumbar spinal cord (Figures 8J and 8K). Thus, Ret is required at step 3, for the formation or extension of third-order interstitial branches of RA mechanoreceptors into the spinal cord.

**DISCUSSION**

We have developed a genetic labeling strategy to show that the early-born Ret+ DRG neurons develop into RA mechanoreceptors, which innervate Meissner corpuscles and Pacinian corpuscles, and form longitudinal lanceolate endings. Interestingly, these RA mechanoreceptors innervate discrete target zones within the gracile and cuneate nuclei, revealing a modality-specific pattern of mechanosensory neuron axonal projections within the brainstem. In addition, we show that NRTN-GFRα2/Ret signaling is essential for development of Pacinian corpuscles, and, importantly, Ret signaling mediates the formation of central connections of most if not all RA mechanosensory neurons in the spinal cord and medulla. Thus, Ret signaling orchestrates the assembly of RA mechanosensory neurons into circuits that underlie tactile discrimination and the perception of touch.
Figure 8. Ret Is Required Autonomously for the Formation of the Third Branch of Central RA Mechanosensory Axons

(A and B) Dil labeling of thoracic DRGs of E15.5 RetERT2;Tau(mGFP) and RetERT2;CFP mice. Consistent with published findings (Ozaki and Snider, 1997), mechanoreceptors have already extended central axonal projections to spinal cord layers III through V at E15.5.

(C) Illustration of RA mechanoreceptor central projections, which can be subdivided into four steps. This illustration is adapted from Brown (1981).

(D) Whole-mount anti-GFP staining of a control RetERT2;Tau(mGFP) DRG from an animal treated with 0.6 mg of 4-HT. Note that the first order central projections are visible in the DRG. On average, 6 ± 2 GFP+ neurons are labeled per thoracic DRG (12 DRGs in total, n = 3 from two separate litters).

(E) Whole-mount anti-GFP staining of a RetERT2;CFP DRG from an animal treated with 1 mg of 4-HT. Note that first order central projections are visible in the DRG. On average, 11 ± 4 CFP+ neurons are labeled per thoracic DRG (20 DRGs in total, n = 4 from three separate litters).

(F) Quantification of labeled DRG neuron number. Error bars show standard error.

(G) Anti-GFP staining of sagittal thoracic spinal cord sections of RetERT2;Tau(mGFP) mice. Note that the third order axonal branches (white arrows) originate from rostral-caudal running fibers and penetrate the spinal cord. Asterisk indicates the position of blood vessels that are autofluorescent and seen in mice of both genotypes.

(H) Anti-GFP staining of sagittal thoracic spinal cord sections of RetERT2;CFP mice. Note that very few third order axonal branches originating from rostral-caudal running fibers are found in these mice.

(I) Quantification of the number of third order axonal branches for the two genotypes. On average, 1.48 ± 0.41 third order axonal branches are observed in every 200 μm of rostral-caudal fiber in RetERT2;Tau(mGFP) control mice (n = 3 from two litters), whereas 0.19 ± 0.05 third order axonal branches are observed in every 200 μm of rostral-caudal fiber in RetERT2;CFP mutant mice (n = 4 from three litters). Error bars show standard error.

(J and K) Anti-GFP staining using horizontal lumbar spinal cord sections taken from RetERT2;Tau(mGFP) (J) and RetERT2;CFP (K) mice. Similar to that observed using sagittal thoracic sections, there are several GFP+ central projections in RetERT2;Tau(mGFP) control mice at this age, but almost no CFP+ central projections are found in the RetERT2;CFP mice.
Modality-Specific Segregation of Mechanosensory Axons in the DC-Medial Lemniscal Pathway

DRG mechanoreceptors send axonal branches to local circuit neurons located in lamina III through V of the spinal cord (Brown, 1981) as well as on second order projection neurons located in the cuneate and gracile nuclei of the medulla. The DCNs, in turn, convey somatosensory information to the thalamus and somatosensory cortex (Carpenter and Sutin, 1983).

Although it is generally appreciated that topographic maps are represented within each of the projections of somatosensory pathways, from the spinal cord to the somatosensory cortex, less is known about modality maps in which RA and SA mechanosensory information is segregated. Interestingly, physiological recordings have pointed to the existence of distinct RA and SA zones along the entire extent of the dorsal-column-medial lemniscal pathway (Dykes, 1983; Dykes et al., 1982; Mountcastle, 1957, 1984; Rasmussen and Northgrave, 1997). Our findings show that afferents of GFP-labeled early Ret+ neurons almost completely fill the gracile nucleus and this nucleus is remarkably smaller in Ret mutant animals, suggesting that RA mechanoreceptors constitute the majority of fibers that innervate the gracile nucleus. Remarkably, the pattern of Ret+ RA mechanosensory afferent terminations within the mouse DCN described in the present study bears striking resemblance to that found by Dykes et al. (1982) using physiological recordings in cats. In addition, our results provide an anatomical basis of “modality re-sorting” (Whitsel et al., 1969), a phenomenon in which RA and SA mechanosensory afferents ascending within the gracile and cuneate fasciculi of the DC reorganize such that RA mechanosensory afferents predominate in the gracile fasciculus at cervical levels. Indeed, our labeling experiments show that Ret+ RA mechanoreceptor afferents are greatly enriched in the cervical gracile fasciculus. The observed enrichment of these RA mechanosensory afferents within the gracile fasciculus of the DC, prior to innervation of the DCN, could provide a mechanistic explanation for the formation of modality maps within the DCN. In fact, a similar mechanism of pre-target sorting of axons has been suggested for the establishment of topographic maps in the olfactory system (Imai et al., 2009). Taken together with previous physiological recordings in the cat, raccoon, and monkey, our genetic labeling results from mice strongly support the existence of modality maps within the DC and DCN and the idea that such maps are found in many mammalian species.

Pacinian Corpuscles Require NRTN-GFRα2/Ret Signaling During Development but Not in Adulthood

We show that development of Pacinian corpuscles is dependent on NRTN, GFRα2, and Ret. Indeed, our finding of a common phenotype in Ntrn, Gfrα2, and Ret<sup>fl/fl</sup>;Wnt1<sup>Cre</sup> mutant mice strongly supports the conclusion that GFRα2 is the Ret co-receptor in RA mechanoreceptors and that a NRTN-GFRα2/Ret signaling cascade in these neurons controls Pacinian corpuscle formation. Our results further show that NRTN-GFRα2/Ret signaling is not required for maintenance of the corpuscle once it has formed. This conclusion is based on the observation that deletion of Ret in 3 week old mice, a time when Pacinian corpuscles are mature (Zelena, 1976), has no obvious effect on corpuscle number or morphology, even in mice up to 6 months of age. In contrast, Ret is required for both development (Luo et al., 2007) and maintenance (this study) of projections of at least one class of Ret+ DRG sensory neurons, the cutaneous endings of non-peptidergic nociceptors. In all, our findings define the first growth factor signaling module required for development of Pacinian corpuscle and show that Ret is differentially required for the maintenance of axonal projections of select classes of Ret+ DRG neurons.

Distinct Roles of Ret and TrkB in the Development of Axonal Projections of Cutaneous RA Mechanoreceptors

In contrast to the severe deficit of Pacinian corpuscles, the phenotypes associated with cutaneous RA mechanosensory end organs of Ret mutant mice are relatively modest. Moreover, consistent with previous studies using TrkB null mutants (Gonzalez-Martinez et al., 2004; Perez-Pinera et al., 2008), we found that TrkB functions within cells of neural crest origin to support formation of Meissner corpuscles, pointing to a role in either the axon, Schwann cell, or both. Since many early Ret+ neurons express TrkB during development, we favor a model in which TrkB signaling within Ret+ RA mechanoreceptors serves to direct the formation of Meissner corpuscles. Alternatively, since Meissner corpuscles can be innervated by either one or two Aβ fibers (Ide, 1976) and Ret and TrkB expression patterns in the DRG do not completely overlap, it is possible that some Meissner corpuscles are innervated by two molecularly distinct subtypes of Aβ fibers, each expressing either Ret or TrkB, and that the TrkB+ fibers instruct the process of corpuscle formation. Either way, it is clear that Ret plays a relatively minor role in the formation of Meissner corpuscles. In striking contrast, Ret is absolutely essential for extension of interstitial branches of central axons of RA mechanoreceptors in the spinal cord, whereas TrkB signaling is dispensable for the formation of these central projections. Therefore, Ret and TrkB signaling differentially support the growth and branching of central axons of RA mechanoreceptors and the formation of cutaneous RA mechanosensory end organs.

It is also interesting to note that Ret is differentially required for development of the central projections of distinct classes of Ret+ DRG neurons. Although dispensable for the central projections of Ret+ non-peptidergic nociceptors (Luo et al., 2007), we show here that Ret plays a critical role in the formation of central projections of RA mechanoreceptors and hence their synaptic connections in the spinal cord and brain stem. Based on this observation, we propose that NRTN and perhaps other Ret ligands may be effective in supporting regeneration of central axonal projections of RA mechanosensory neurons, which could enable functional recovery of tactile discrimination and the perception of touch following nerve or spinal cord damage.

EXPERIMENTAL PROCEDURES

Mouse Lines and Treatments

The details of Ret<sup>HIPI</sup> mouse generation can be found in the Supplemental Experimental Procedures. Ret-conditional CFP mice (Ret<sup>CPF</sup>) were generated as described previously (Uesaka et al., 2008). The Tau-conditional mGFP mouse line (Tau<sup>mGFP</sup>) (Hippemeyer et al., 2005) was generously provided by G. Fishell (New York University) and S. Arber (Biocenter/Friedrich Neuron

RA Mechanoreceptors and Ret Signaling
target innervation. Neuron cutaneous mechanoreceptors require neurotrophin-3 following peripheral
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In Situ Hybridization and Double Fluorescent In Situ Hybridization
Digoxigenin-labeled cRNA probes were used for in situ hybridization. In situ
hybridization probes directed against Gfra1, Gfra2, Gfra3, Ret, TrkA, TrkB, and
RetK were described previously (Luo et al., 2007). Details are found in the
Supplemental Experimental Procedures.

Additional materials and methods can be found in the Supplemental Exper-
imental Procedures.

SUPPLEMENTAL DATA
Supplemental Data include Supplemental Experimental Procedures and 12
figures and can be found with this article online at http://www.cell.com/
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