Development/Plasticity/Repair

A RET-ER81-NRG1 Signaling Pathway Drives the Development of Pacinian Corpuscles

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Axon-Schwann cell interactions are crucial for the development, function, and repair of the peripheral nervous system, but mechanisms underlying communication between axons and nonmyelinating Schwann cells are unclear. Here, we show that ER81 is functionally required in a subset of mouse RET$^+$ mechanosensory neurons for formation of Pacinian corpuscles, which are composed of a single myelinated axon and multiple layers of nonmyelinating Schwann cells, and *Ret* is required for the maintenance of *Er81* expression. Interestingly, *Er81* mutants have normal myelination but exhibit deficient interactions between axons and corpuscle-forming nonmyelinating Schwann cells. Finally, ablating *Neuregulin-1* (*Nrg1*) in mechanosensory neurons results in no Pacinian corpuscles, and an *Nrg1* isoform not required for communication with myelinating Schwann cells is specifically decreased in *Er81*-null somatosensory neurons. Collectively, our results suggest that a RET-ER81-NRG1 signaling pathway promotes axon communication with nonmyelinating Schwann cells, and that neurons use distinct mechanisms to interact with different types of Schwann cells.

Key words: development; ER81; neuregulin 1; neuron Schwann cell interaction; Pacinian corpuscle; RET signaling

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Significance Statement

Communication between neurons and Schwann cells is critical for development, normal function, and regeneration of the peripheral nervous system. Despite many studies about axonal communication with myelinating Schwann cells, mostly via a specific isoform of Neuregulin1, the molecular nature of axonal communication with nonmyelinating Schwann cells is poorly understood. Here, we described a RET-ER81-Neuregulin1 signaling pathway in neurons innervating Pacinian corpuscle somatosensory end organs, which is essential for communication between the innervating axon and the end organ nonmyelinating Schwann cells. We also showed that this signaling pathway uses isoforms of Neuregulin1 that are not involved in myelination, providing evidence that neurons use different isoforms of Neuregulin1 to interact with different types of Schwann cells.

Introduction

The peripheral nervous system (PNS) contains neurons and two broad classes of Schwann cells: myelinating and nonmyelinating

Schwann cells. Myelinating Schwann cells produce the myelin sheath insulating large-diameter axons, whereas nonmyelinating Schwann cells help to form specialized sensory and motor endings and bundle small-diameter unmyelinated sensory neurons (Griffin and Thompson, 2008; Monk et al., 2015). Neurons and Schwann cells interact closely to build the PNS, maintain its normal function, and regenerate the PNS when necessary (Jessen et al., 2015; Salzer, 2015; Taveggia, 2016). Despite extensive investigation of myelinating Schwann cells, relatively little is known about communication between axons and nonmyelinating Schwann cells at present.

To gain a better understanding of axonal communication with nonmyelinating Schwann cells, we investigated the development of Pacinian corpuscles in mice. Pacinian corpuscles are large mechanosensory end organs, which sense high-frequency vibration and are classified as rapidly adapting (RA) mechanore-

Figure 1. *Er81* is expressed in nonmyelinating Schwann cells of Pacinian and Meissner corpuscles. *A*, Nuclear *LacZ* driven from the *Er81* locus labels the nuclei of *Er81*^{+} cells. Anti-LacZ (green) immunostaining combined with anti-S100 (red) staining shows *Er81* expression in the inner core Schwann cells of Pacinian corpuscles in whole-mount staining of the interosseous membrane at P7. *B*, Anti-LacZ and anti-S100 immunostaining of hindpaw glabrous skin sections from P21 *Er81*[LacZ/+] mice shows expression of *Er81* in the Schwann cells of Meissner corpuscle end organs. *C*, *D*, Anti-S100 and anti-NFH staining of paw glabrous skin sections from P21 *Er81*[LacZ/+] control and *Er81*[LacZ/LacZ] null mice reveals no obvious deficit in the formation or innervation of Meissner corpuscles in *Er81*-nulls relative to controls. *N* = 3 animals per genotype. Scale bars: *A*, *C*, *D*, 20 μm; *B*, 5 μm.

ceptors based on their response to sustained stimuli (Zelená, 1994; Fleming and Luo, 2013). Each Pacinian corpuscle is innervated by a single myelinated RA mechanosensory axon. Upon entering the corpuscle, the axon loses its contact with myelinating Schwann cells and directly contacts the corpuscle inner core non-myelinating Schwann cells (Pease and Quilliam, 1957). Therefore, Pacinian corpuscles present an ideal system to investigate axonal interactions with both myelinating and nonmyelinating Schwann cells.

RA mechanoreceptor innervating neurons develop from DRG neurons, which express the receptor tyrosine kinase *Ret* during early embryonic development (Bourane et al., 2009; Luo et al., 2009; Honma et al., 2010). In *Ret* mutant mice, Pacinian corpuscles do not form, whereas the other RA mechanoreceptor endings, such as Meissner corpuscles and lanceolate endings, are still present (Luo et al., 2009). Pacinian corpuscles are also absent in mice lacking the ETS transcription factor *Er81* (or *Etv1*) (Sedý et al., 2006), which controls the central projections and end organ development of proprioceptors in the PNS (Arber et al., 2000; Patel et al., 2003; de Nooij et al., 2013). Because *Er81* is expressed in corpuscle-forming Schwann cells, the loss-of-Pacinian corpuscle phenotype was previously attributed to the loss of *Er81* function in Schwann cells (Sedý et al., 2006). However, ER81 is also expressed in nonproprioceptive DRG neurons (Arber et al., 2000), raising the possibility that *Er81* may be expressed and function in Pacinian corpuscle-innervating neurons.

Neuregulin-1 (NRG1) is one major effector of axon-Schwann cell communication in the PNS (Birchmeier and Nave, 2008). Isoforms of *Nrg1* containing a cysteine-rich domain (*Nrg1-CRD*) are crucial for axonal interactions with myelinating Schwann cells (Michailov et al., 2004). In contrast, *Nrg1* isoforms with Ig-like domains (*Nrg1-Ig*) are required in neurons for the formation of proprioceptive muscle spindles (Hippenmeyer et al., 2002; Cheret et al., 2013). Given that Pacinian corpuscle-innervating axons are highly myelinated and that their end organs are formed by axons and nonmyelinating Schwann cells, it is possible that NRG1 may have unknown important roles in Pacinian corpuscle development.

Here, we show that *Er81* is expressed in limb RA mechanoreceptor neurons and required in neurons for Pacinian corpuscle development. The maintenance of *Er81* expression depends on *Ret*, but not vice versa. Interestingly, *Er81* is likely required for communication between axons and nonmyelinating Schwann cells of Pacinian corpuscles but dispensable for myelination. Finally, we generated mechanosensory neuron-specific mutants of *Nrg1* and found that Pacinian corpuscles are not formed in these mutant mice. Because the expression of *Nrg1-Ig*, but not *Nrg1-CRD*, is decreased in *Er81* mutant mice, ER81 may regulate interactions between axons and nonmyelinating Schwann cells via NRG1-Ig. Collectively, our study establishes a RET-ER81-NRG1 pathway in RA mechanoreceptors for specifying Pacinian corpuscles during development and identifies ER81 as a potential transcriptional regulator of *Nrg1*. Our results also suggest that neurons use different isoforms of NRG1 to interact with different types of Schwann cells.

Materials and Methods

Mouse strains. All mice, except for surgical animals, *Bace1*, and *Nrg1-CRD* mice, were raised in a barrier facility in Hill Pavilion at the University of
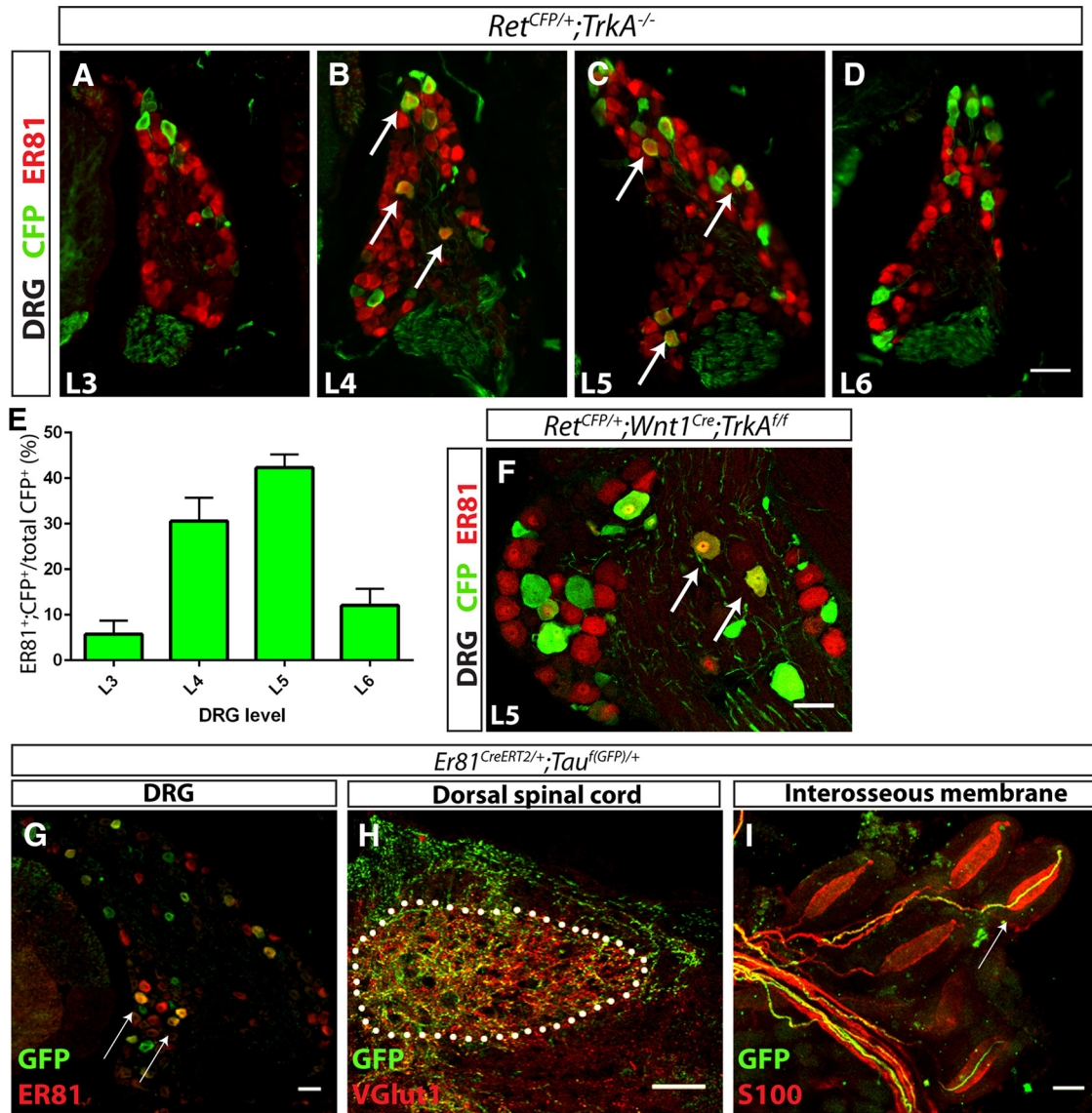
Figure 2. ER81 is expressed in Pacinian corpuscle-innervating neurons. ***A–E***, Anti-CFP (green) and anti-ER81 (red) immunostaining of distal hindlimb-innervating (L4 and L5) and neighboring (L3 and L6) DRG sections of P0 $Ret^{CFP/+}$; $TrkA^{-/-}$ mice. A high degree of ER81 expression is present in distal limb-innervating RA mechanoreceptors relative to nondistal limb-innervating RA mechanoreceptors (5.71 ± 2.43% of L3, 30.57 ± 4.19% of L4, 42.30 ± 2.37% of L5, and 12.08 ± 2.98% of L6 RA mechanoreceptors express ER81). ***F***, At P15, 13.5 ± 1.3% of CFP $^+$ RA mechanoreceptors in L5 DRGs express ER81 in a $Ret^{CFP/+}$; $Wnt1^{Cre}$; $TrkA^{f/f}$ mice. Arrows indicate double positive DRG neurons. ***G–I***, $Er81^{ERT2/+}$; $Tau^{f(GFP)/+}$ mice were treated with tamoxifen at E14.5 and E15.5, permanently labeling a subset of $Er81^+$ cells with GFP, and analyzed at P15. ***G***, Anti-GFP (green) and anti-ER81 (red) immunostaining of DRG sections shows that a subset of ER81 $^+$ neurons express GFP. ***H***, Dorsal spinal cord immunostaining with anti-VGLUT1 (red), which labels presynaptic terminals of mechanosensory neurons, reveals that GFP $^+$ neurons innervate mechanosensory-specific layers III-V of the dorsal spinal cord. ***I***, Whole-mount anti-S100 (red) and anti-GFP staining of interosseous membrane shows Pacinian corpuscles innervated by GFP $^+$ fibers, as indicated by a white arrow. $N = 3$ animals per genotype. Scale bar, 50 μm. Error bars indicate SEM.

Pennsylvania. Surgical animals were maintained in a conventional facility in John Morgan Building at the University of Pennsylvania. *Bace1* mice were raised at the Johns Hopkins mouse facility, and *Nrg1-CRD* mice were raised at the mouse facility of the State University of New York at Stony Brook. All mice, except *Bace1* and *Nrg1-CRD* strains, were maintained on a mixed c57 bl/6j and CD1 background. *Bace1* and *Nrg1-CRD* mice were maintained on a c57 bl/6j background. All procedures were conducted according to animal protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and National Institutes of Health guidelines. Previously described mouse lines include the following: $Er81^{LacZ}$ (Arber et al., 2000) (RRID:MGI: 2384496), $Wnt1^{Cre}$ (Danielian et al., 1998) (RRID: IMSR_JAX:003829), $TrkA^-$ (Fleming et al., 2015), $TrkA^f$ (Chen et al., 2005) (RRID:IMSR_JAX:022362), Ret^{CFP} (Uesaka et al., 2008) (RRID:MGI: 3777556), $Er81^{CreERT2}$ (Taniguchi et al., 2011) (RRID:MGI:4838417), $Tau^{f(GFP)}$ (Hippenmeyer et al., 2005) (RRID:MGI:3590682), $Nestin^{Cre}$ (Tronche et al., 1999) (RRID:MGI:2176173), $Er81^f$ (Patel et al., 2003)

(RRID:MGI:2663693), *ROSA-tdTomato* (Madisen et al., 2010) (RRID:MGI: 3809523), Dhh^{Cre} (Jaegle et al., 2003) (RRID:MGI:4359600), Bax^- (Knudson et al., 1995) (RRID:MGI:1857429), $Ret^{CreERT2}$ (Luo et al., 2009) (RRID: MGI:4437245), $Nrg1^f$ (Zhang et al., 2011) (RRID:MGI:5292107), *Nrg1-CRD$^-$* (Wolpowitz et al., 2000) (RRID:MGI:1928831), and $Bace1^-$ (Savonenko et al., 2008) (RRID:MGI:2389228). The $Nrg1^-$ allele was generated by crossing an $Nrg1^{f/+}$ male to a $Sox2^{Cre}$ female (RRID:IMSR_JAX:008454).

Tissue preparation, histology, and in situ *hybridization.* For immunostaining of spinal columns and DRGs, mice were anesthetized with a mixture of ketamine, xylazine, and acerpromazine by intraperitoneal injection. Mice were then transcardially perfused with PBS, followed by perfusion with 4% PFA in PBS. Spinal columns were then dissected and postfixed in 4% PFA in PBS for 2–4 h at 4°C, followed by overnight cryoprotection in 30% sucrose in PBS at 4°C. In mice processed only for leg and skin tissue for cryosectioning, mice were killed with CO_2 followed by cervical
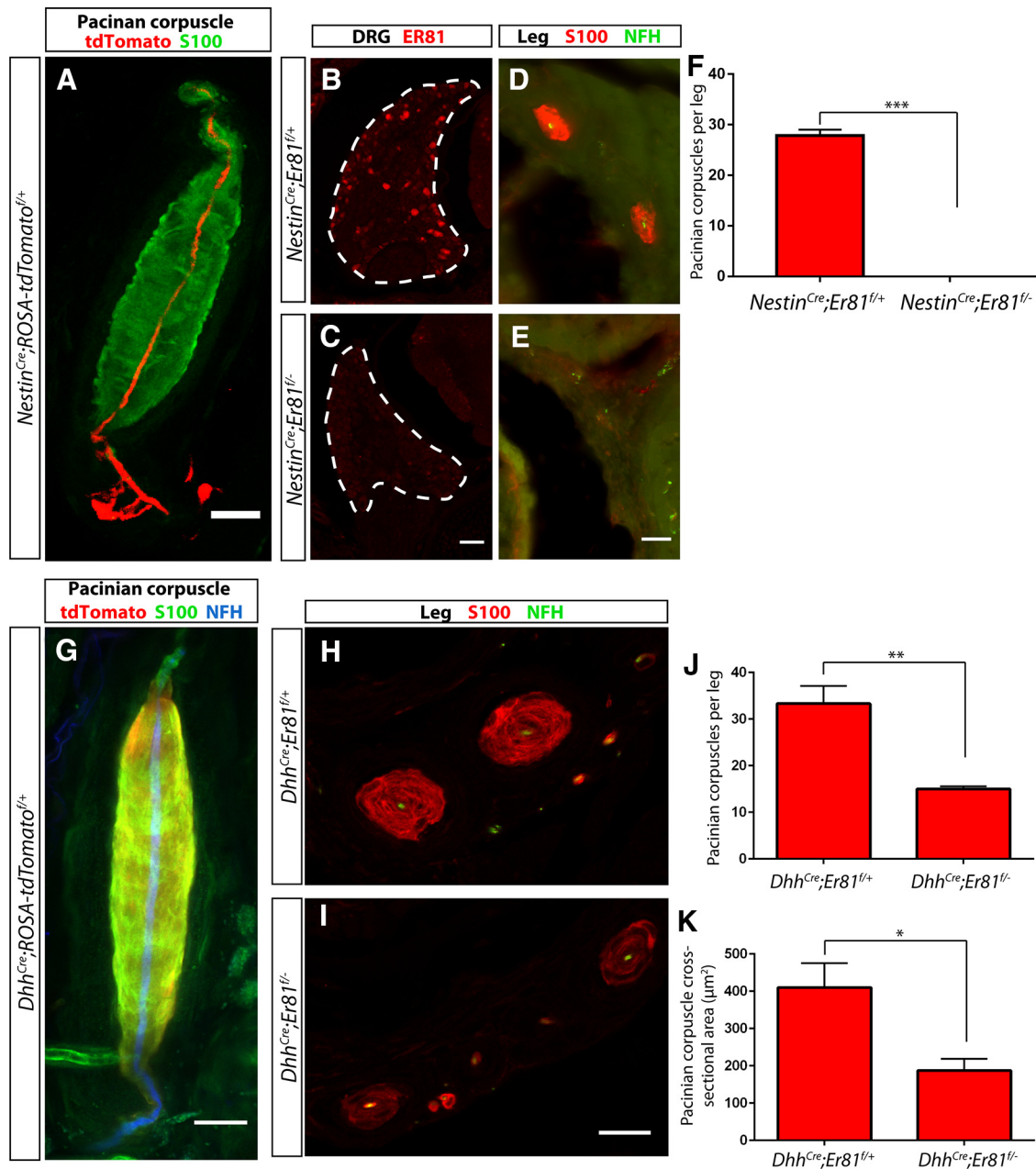
Figure 3. *Er81* is absolutely required in neurons, but not Schwann cells, for Pacinian corpuscle development. ***A***, *Nestin*^Cre crossed to a *ROSA-tdTomato* (red) reporter allele exhibits recombination in innervating fibers but not in the S100 ^+ (green) Schwann cells of the Pacinian corpuscle end organ. ***B***, ***C***, Anti-ER81 immunostaining of DRG sections from P7 *Nestin*^Cre*;Er81*^f/+ control (***B***) and *Nestin*^Cre*;Er81*^f/− conditional mutant (***C***) shows ablation of ER81 expression in neurons in the mutant mice. ***D–F***, Anti-S100 staining of hindlimb sections from P7 control (***D***) and conditional mutant (***E***) mice shows a lack of S100 ^+ Pacinian corpuscles surrounding the fibula in neural-specific mutant mice (27.83 ± 1.167 PC per leg in *Nestin*^Cre*;Er81*^f/+ control, 0 ± 0 PC per leg in *Nestin*^Cre*;Er81*^f/− conditional mutant; $p < 0.0001$). ***G***, *Dhh*^Cre crossed to a *ROSA-tdTomato* (red) reporter allele reveals reporter activity in S100 ^+ (green) inner core Schwann cells of the Pacinian corpuscles, but not in the NFH ^+ (blue) innervating fiber. ***H***, ***I***, Pacinian corpuscles are present around the fibula in *Dhh*^Cre*;Er81*^f/+ control (***H***) and *Dhh*^Cre*;Er81*^f/− conditional mutant (***I***) mice at P7. ***J***, Quantification of serial leg sections reveals a significant decrease in the number of Pacinian corpuscles per leg in Schwann cell-specific *Er81* mutants compared with controls (33.33 ± 3.756 PC per leg in *Dhh*^Cre*;Er81*^f/+ controls, 15.00 ± 0.58 PC per leg in *Dhh*^Cre*;Er81*^f/− conditional mutants; $p = 0.009$). ***K***, The average size of Pacinian corpuscles, measured by cross-sectional area, is significantly reduced in Schwann cell-specific *Er81* mutants (410.1 ± 65.3 μm² in *Dhh*^Cre*;Er81*^f/+ controls, 187.9 ± 30.9 μm² in *Dhh*^Cre*;Er81*^f/− conditional mutants; $p = 0.037$). $N = 3$ animals, 6 legs per genotype. Scale bars: ***A***, ***D–I***, 20 μm; ***B***, ***C***, 50 μm. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Error bars indicate SEM.

dislocation/decapitation. Paw skin was fixed overnight in 4% PFA in PBS at 4°C, followed by cryopreservation. Legs were fixed 2–4 h in 4% PFA in PBS at 4°C, and then decalcified overnight in 22% formic acid and 10% sodium citrate in ddH₂O at room temperature (Luo et al., 2009), followed by cryopreservation. Preserved tissue was then embedded in NEG-50 and sectioned at 20 μm (spinal columns/DRGs), 30–40 μm (leg/skin tissue for immunostaining), or 10–15 μm (leg tissue for H&E staining). For spinal columns, care was taken in the embedding and sectioning process to ensure collection of specific lumbar DRG levels. Serial leg sections from ankle to knee were collected to ensure collection of all Pacinian corpuscles. Cryosection immunostaining was performed as previously reported (Fleming et al., 2015).

Whole-mount interosseous membranes were dissected from hindlimbs following above perfusion procedure and then postfixed for 2–4 h as above. Following fixation, whole-mount membranes were permeabilized 3 × 10 min with PBS with 1% Triton X-100 and 1% Tween 20. Antibody incubation was performed overnight at room temperature with rocking in PBS plus 5% lamb serum, 1% Triton X-100, and 1% Tween 20. The following day, sections were washed 3 × 1 h with PBS plus
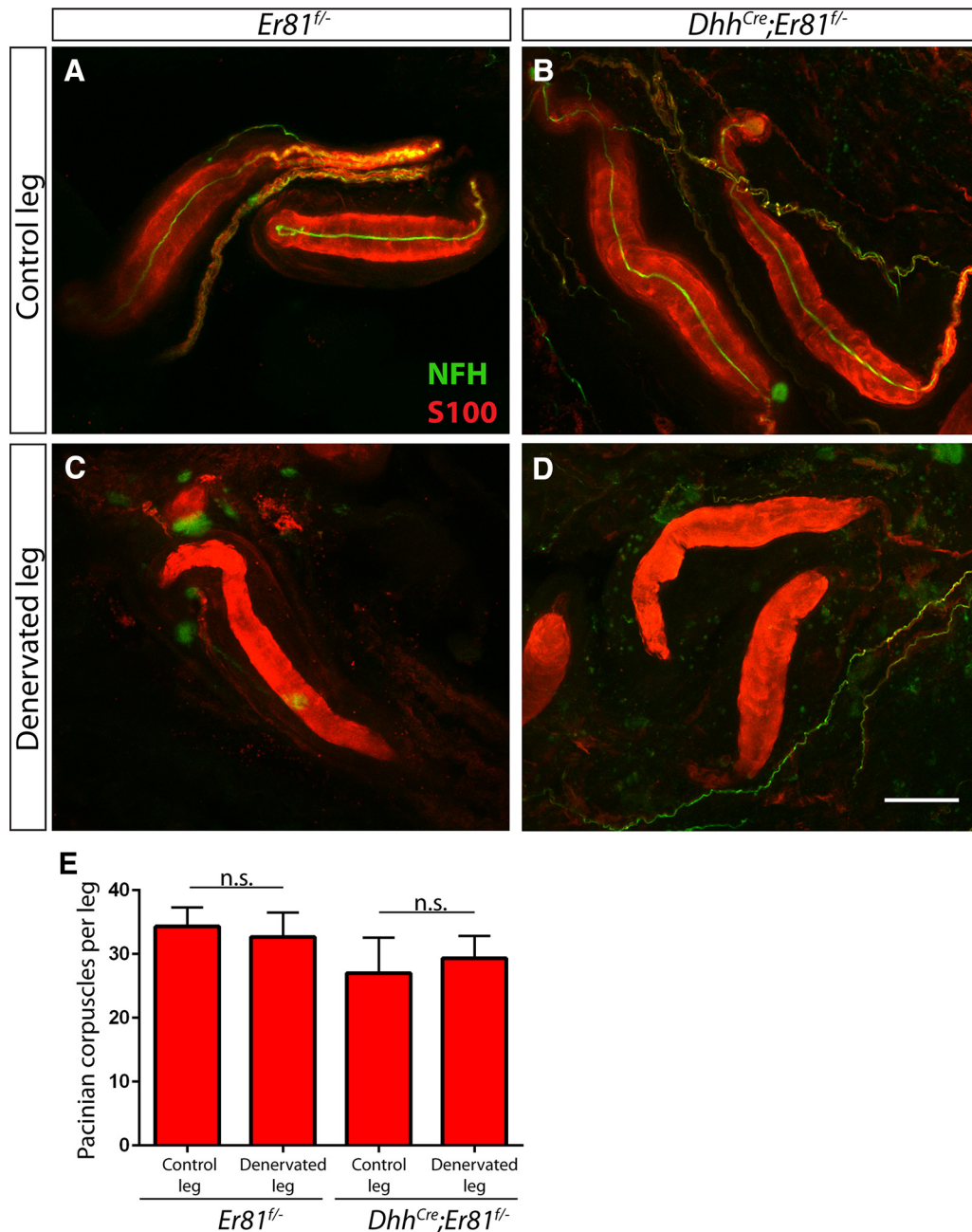
Figure 4. *Er81* in Schwann cells is not required for maintenance of Pacinian corpuscles following denervation. ***A–D***, Two- to 4-month-old *Er81*$^{f/-}$ control (***A***, ***C***) and *Dhh*^{Cre};*Er81*$^{f/-}$ conditional mutants (***B***, ***D***) underwent unilateral tibial nerve transection and recovered for 2 weeks before tissue processing. Denervation was confirmed by the loss of NFH$^+$ (green) axons innervating S100$^+$ (red) Pacinian corpuscles in whole-mount interosseous membrane staining of the denervated limb (***C***, ***D***), in contrast to NFH$^+$ innervation in the nonsurgical limb (***A***, ***B***). ***E***, Quantification of S100$^+$ Pacinian corpuscles in serial hindlimb sections in *Er81*$^{f/-}$ controls (34.33 ± 2.96 corpuscles per nonsurgical limb, 32.67 ± 3.84 corpuscles per denervated limb; $p = 0.75$) and *Dhh*^{Cre}; *Er81*$^{f/-}$ conditional mutants (27.00 ± 5.57 corpuscles per nonsurgical limb, 29.33 ± 3.53 corpuscles per denervated limb; $p = 0.74$) shows that loss of *Er81* in Schwann cell does not cause a deficit in the maintenance of Pacinian corpuscles following denervation. $N = 3$ legs per condition. Not significant: $p \geq 0.05$. Error bars indicate SEM. Scale bar, 50 μm.

1% Tween 20. Secondary antibody incubation was performed overnight at room temperature with rocking in PBS plus 5% lamb serum and 1% Tween 20. Samples were then washed 3 × 1 h in PBS plus 1% Tween 20, and then cleared in 50% glycerol in PBS for 30 min, followed by 75% glycerol in PBS for 30 min, and then mounted and imaged in 75% glycerol in PBS.

Antibodies used were as follows: chicken anti-LacZ (1:500, Aves Laboratories catalog #BGL-1040 RRID:AB_2313507), chicken anti-GFP (1:500, Aves Laboratories catalog #GFP-1020 RRID:AB_10000240), chicken anti-neurofilament heavy-chain (NFH) (1:500, Aves Laboratories catalog #NF-H RRID:AB_2313552), guinea pig anti-VGLUT1 (1: 1000, Millipore catalog #AB5905 RRID:AB_2301751), rabbit anti-S100

(1:400, Dako catalog #Z031129−2 RRID:AB_2315306), rabbit anti-ER81 (1:1000) (Arber et al., 2000) (RRID:AB_2617167), and AlexaFluor-conjugated goat or donkey raised secondary antibodies (1:500, Invitrogen or Jackson ImmunoResearch Laboratories). H&E staining was performed as previously described (Luo et al., 2009). *In situ* hybridization was performed as previously described (Fleming et al., 2015).

For electron microscopy studies, anesthetized mice were transcardially perfusion fixed with 2% PFA and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The tissues were quickly removed, then postfixed in perfusing solution for at least 4 h at 4°C. Nerves were osmicated and dehydrated. They were treated twice with propylene oxide for 5 min, incubated sequentially with Embed 812:propylene oxide mixtures (1:1
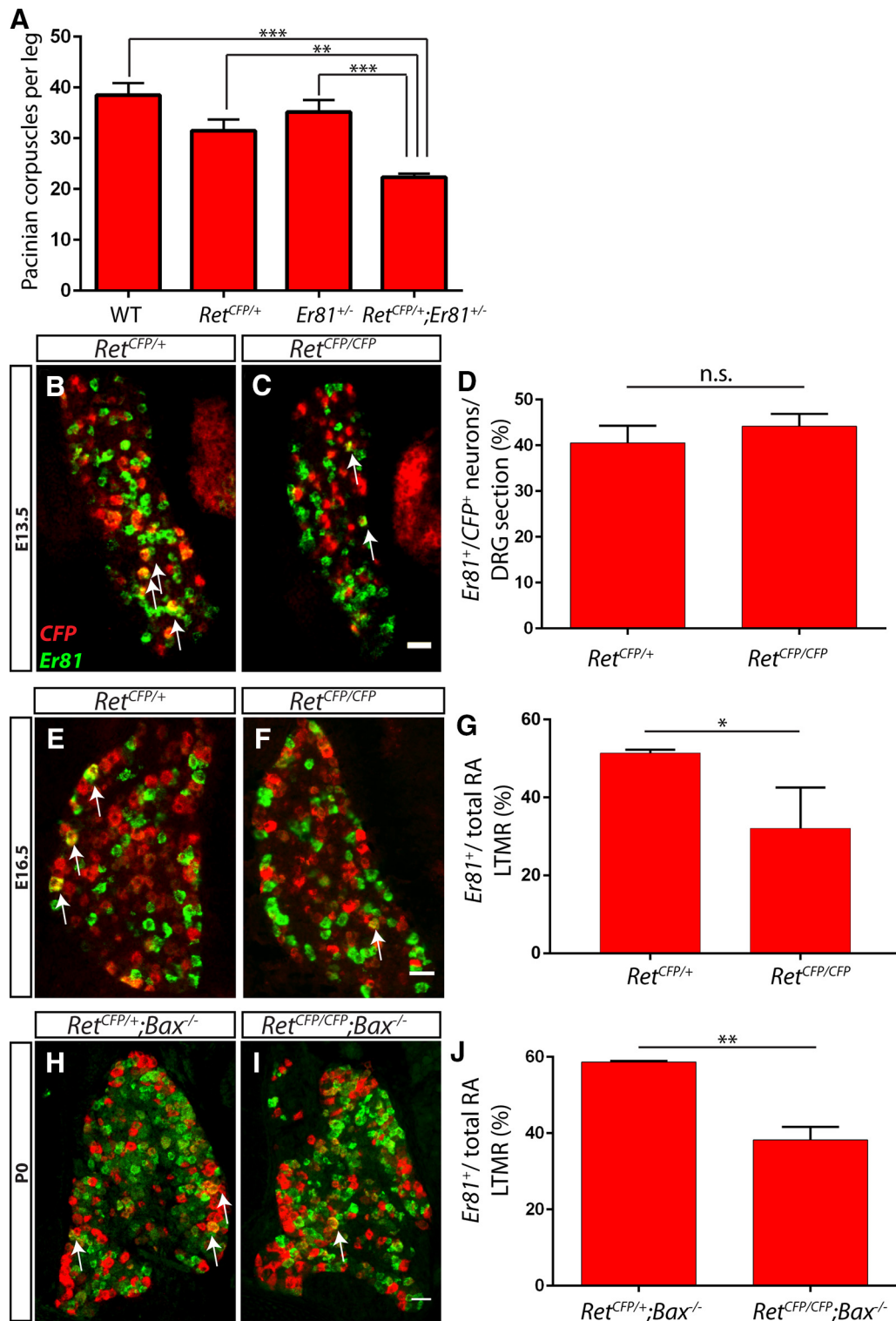
Figure 5. *Ret* is required for the maintenance, but not initiation, of *Er81* expression in RA mechanoreceptors. *A*, The number of S100 $^+$ Pacinian corpuscles per limb was quantified in serial sections of P14–P21 wild-type (38.50 ± 2.36 Pacinian corpuscles per limb), *Ret$^{CFP/+}$* (31.50 ± 2.23 Pacinian corpuscles per limb), *Er81$^{+/-}$* (35.17 ± 2.40 Pacinian corpuscles per limb), and *Ret$^{CFP/+}$*; *Er81$^{+/-}$* (22.33 ± 0.71 Pacinian corpuscles per limb) hindlimbs. The number of Pacinian corpuscles in *Ret$^{CFP/+}$*;*Er81$^{+/-}$* double heterozygotes is significantly less than in all other genotypes. *B–D*, Double-fluorescent *in situ* hybridization against *CFP* (red) driven from the *Ret* locus and *Er81* (green) in L4/L5 DRGs of *Ret$^{CFP/+}$* and *Ret$^{CFP/CFP}$* embryos at E13.5. The percentage of *CFP$^+$* neurons expressing *Er81* is similar between controls and mutants (40.55 ± 3.74% in *Ret$^{CFP/+}$* DRGs, 44.19 ± 2.65% in *Ret$^{CFP/CFP}$* DRGs; *p* = 0.47). *E–G*, At E16.5, *CFP/Er81* double-fluorescent *in situ* hybridization shows a decrease in the proportion of RA mechanoreceptors that express *Er81* in *Ret* mutants relative to controls (51.40 ± 0.50% in *Ret$^{CFP/+}$* DRGs, 32.12 ± 6.03% in *Ret$^{CFP/CFP}$* DRGs; *p* = 0.03). *H–J*, *CFP/Er81* double-fluorescent *in situ* hybridization of P0 *Ret* mutants and controls in an apoptosis-deficient *Bax*-null background shows a significant decrease in the percentage of RA mechanoreceptors that express *Er81* in *Ret* mutants (58.69 ± 0.26% in *Ret$^{CFP/+}$*;*Bax$^{-/-}$* DRGs, 38.24 ± 3.40% in *Ret$^{CFP/CFP}$*; *Bax$^{-/-}$* DRGs; *p* = 0.004). *N* = 6 limbs (*A*), 3 animals (*B–G*), or 2 animals (*H–J*) per genotype. Scale bar, 50 μm. **p* < 0.05. ***p* < 0.01. ****p* < 0.001. Not significant, *p* > 0.05. Error bars indicate SEM.
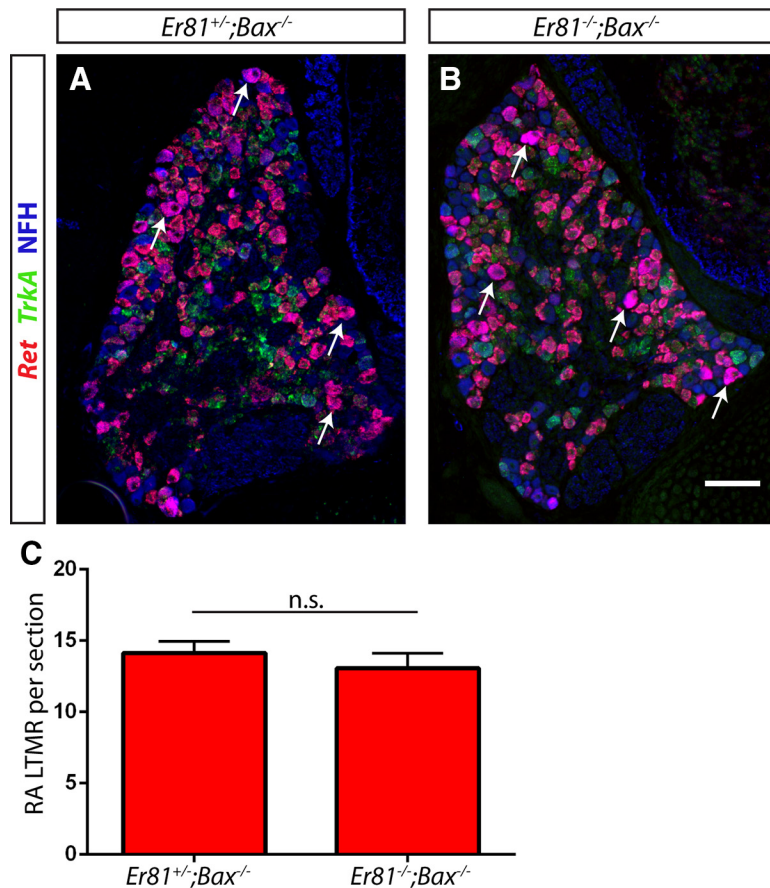
Figure 6. *Er81* does not affect the expression of *Ret* in RA mechanoreceptors. *A–C*, The number of *Ret*$^+$;*TrkA*$^-$;NFH$^+$ RA mechanoreceptors per L4/L5 DRG section was similar between *Er81* mutants and controls in an apoptosis-deficient background at P3–P5, suggesting that *Er81* does not regulate the expression of *Ret* in RA mechanoreceptors (14.12 ± 0.82 RA mechanoreceptors per section in *Er81*$^{+/-}$;*Bax*$^{-/-}$ controls, 13.06 ± 1.06 RA mechanoreceptors per section in *Er81*$^{-/-}$;*Bax*$^{-/-}$ double mutants; *p* = 0.47). Arrows indicate some *Ret*$^+$;*TrkA*$^-$;NFH$^+$ neurons. *N* = 3 animals per condition, ≥8 sections per animal. Not significant: *p* ≥ 0.05. Error bars indicate SEM. Scale bar, 100 μm.

and 2:1), and incubated in pure Embed 812 mixture (Electron Microscopy Sciences) overnight. Tissues were then embedded in fresh Embed 812 mixture at 60°C for 48 h. For light microscopy, 1-μm-thick, transverse sections were stained with alkaline toluidine blue. For electron microscopy, 0.1 μm thick, transverse sections were stained with lead citrate.

Image acquisition. Fluorescent images were collected on a Leica SP5II confocal microscope and a Leica DM5000B microscope. Bright-field images were collected with a Leica DM5000B microscope. Electron micrographs were collected on a Jeol-1010 transmission electron microscope.

Animal surgery. Two- to 4-month-old mice were anesthetized with the inhalation of ~3% isoflurane in an induction chamber and then maintained on 1%–2% isoflurane through a nose cone throughout the procedure. Before making the surgical incision, a very small volume (no >2 mg/kg) of bupivacaine (0.25%) was injected with a 25 gauge needle at equidistant places ~0.5–1 cm apart, in an ellipse around the incision site. Following incision, the overlying muscle was dissected and the right tibial nerve was doubly ligated with 5-O silk threads, and transected between the ligations with iridectomy scissors. The proximal and distal ends were separated by a gap and attached to an adjacent muscle with a suture. The skin was then sutured with 5-O silk threads. Following surgery, mouse was given subcutaneous injection of ~1 ml of lactated Ringer's solution to prevent dehydration and was kept on a heating pad until fully recovered from anesthesia. Meloxicam (2 mg/kg, s.c.) was given at the time of anesthesia induction and every 12 h for 2 d after surgery. Mice were then processed for collection of leg tissue 2 weeks to 3 months following surgery as above.

Tamoxifen treatment. Timed pregnancies were set up in the evening, and female mice were checked for vaginal plug the following morning. The time that vaginal plug was observed was considered embryonic day 0.5 (E0.5). Pregnant dams carrying the *Er81*^{CreERT2} embryos were treated with 10 mg of tamoxifen dissolved in sunflower oil by oral gavage at E14.5 and E15.5. Pregnant dams carrying the *Ret*^{CreERT2} embryos were treated with 5 mg of tamoxifen, 5 μg β-estradiol, and 2.5 mg progesterone dissolved in sunflower oil by oral gavage at E13.5 and E14.5.

Quantification and statistics. For histological analysis of DRG neurons, at least 6 sections per animal were quantified. Image names were randomized after collection to blind experimenter to genotype of images. Pacinian corpuscle counts were performed by manually scanning slides of anti-S100/anti-NFH-stained serial cryosections. Pacinian corpuscles, that appeared at similar locations in consecutive sections were not double counted. Pacinian corpuscle size measurements were obtained by imaging every fifth serial section, to avoid double counting individual corpuscles, which span multiple sections. The area of individual corpuscle cross sections was then measured by manually tracing the S100$^+$ corpuscle inner cores in ImageJ. G-ratios were calculated by manually measuring the area of individual fibers and their axons in ImageJ, and then calculating the diameter of fibers based on the assumption that the measured areas corresponded to a circle. *p* values were calculated by unpaired *t* tests after finding the mean measurement for each individual animal. Statistical analysis was performed with GraphPad Prism software.

Results

Er81 is required for the development of Pacinian, but not Meissner, corpuscles

Pacinian corpuscles and their innervating axons are absent in *Er81* mutant mice around birth (Sedý et al., 2006), but how ER81 contributes to Pacinian corpuscle formation is unclear. The lack of Pacinian corpuscles in *Er81* mutants may be due to its function in Schwann cells, as *Er81* is expressed in the inner core nonmyelinating Schwann cells of Pacinian corpuscles (Sedý et al., 2006). To test this idea, we examined whether *Er81* is specifically expressed in Pacinian corpuscle nonmyelinating Schwann cells or broadly expressed in other end organ Schwann cells of RA mechanoreceptors, such as in Meissner corpuscles (Zelená, 1994; Fleming and Luo, 2013). Using an *Er81*^{LacZ} reporter allele (Arber et al., 2000), we found that *Er81* is expressed in Pacinian (Sedý et al., 2006) as well as Meissner corpuscle inner core Schwann cells (Fig. 1*A*,*B*), suggesting that *Er81* is not specifically expressed in Pacinian corpuscle inner core Schwann cells. Next, we asked whether *Er81* is also required for Meissner corpuscle formation during development. We confirmed the complete absence of Pacinian corpuscles in *Er81*-null mice (Sedý et al., 2006) (data not shown). However, when we examined Meissner corpuscles of postnatal day 21 (P21) control and *Er81*-null mice, we found that they are normal in the mutant dermal papillae (Fig. 1*C*,*D*). Thus, like *Ret*, *Er81* is required for the development of Pacinian corpuscles but not Meissner corpuscles. This result suggests that *Er81*
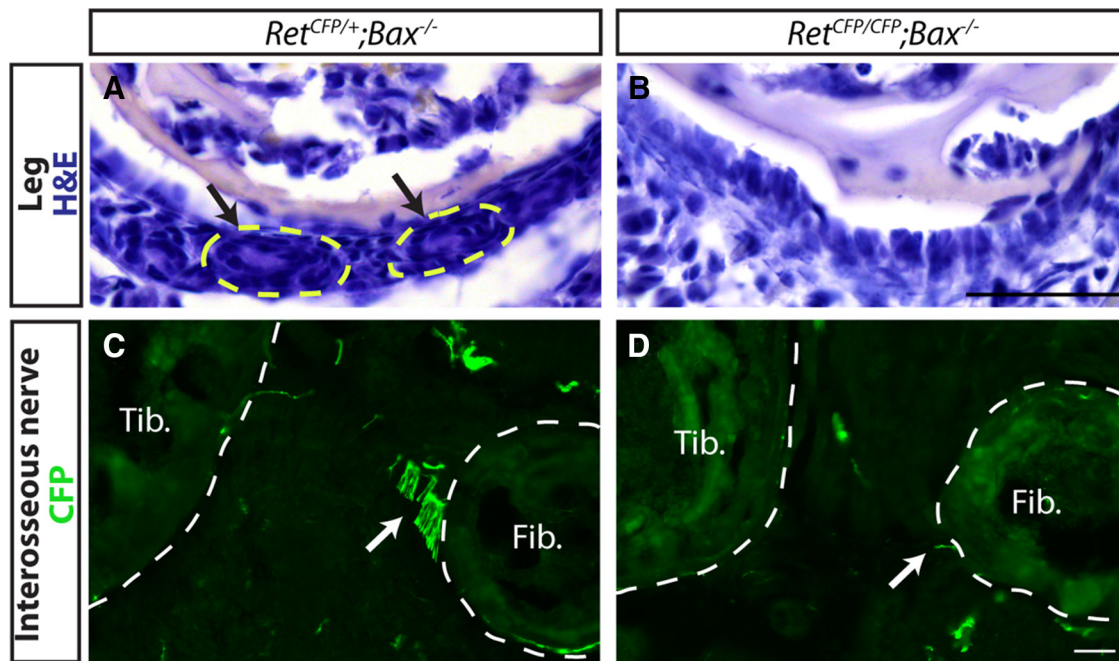
Figure 7. Cell death independent phenotype of *Ret*-null Pacinian corpuscles. *A*, *B*, H&E-stained sections of P0 *Ret$^{CFP/+}$;Bax$^{-/-}$* control (*A*) and *Ret$^{CFP/CFP}$;Bax$^{-/-}$* double-mutant (*B*) hindlimbs. Arrow indicates rudimentary Pacinian corpuscles, which are present in the *Ret$^{CFP/+}$;Bax$^{-/-}$* control but not *Ret$^{CFP/CFP}$;Bax$^{-/-}$* double-mutant hindlimbs. *C*, *D*, Anti-CFP staining of P0 *Ret$^{CFP/+}$; Bax$^{-/-}$* and *Ret$^{CFP/CFP}$;Bax$^{-/-}$* hindlimbs, which labels the axons of RA mechanoreceptors. Arrow indicates axons of interosseous nerve, which are absent in double mutants. T, Tibia; F, fibula. *n* = 2 animals per genotype. Scale bar, 50 μm.

may act in a cell type other than Schwann cells to promote Pacinian corpuscle end organ formation.

Er81 is expressed in Pacinian corpuscle-innervating neurons

To determine whether ER81 functions in Pacinian corpuscle-innervating neurons, we first asked whether ER81 is expressed in RA mechanoreceptors at distal limb-innervating levels, where Pacinian corpuscle-innervating neurons are mainly found. We quantified the proportion of RA mechanoreceptors that are immunopositive for ER81 in P0 *Ret$^{CFP/+}$;TrkA$^{-/-}$* DRG sections. In this genetic background, all *Ret$^+$* neurons were identified with anti-GFP immunostaining. Additionally, because all *Ret$^+$* DRG neurons in addition to RA mechanoreceptors depend on NGF/TrkA signaling for their survival and expression of *Ret* (Luo et al., 2007; Luo et al., 2009), most CFP$^+$ neurons in these sections should be RA mechanoreceptors. Using this strategy, we found that ~40% of RA mechanoreceptors in distal hindlimb-innervating DRGs (L4 and L5) express ER81, whereas only ~10% of RA mechanoreceptors in surrounding DRGs (L3 and L6) express ER81 (Fig. 2*A–E*). Therefore, ER81 is enriched in distal hindlimb-innervating RA mechanoreceptors. To determine whether limb RA mechanoreceptors maintain expression of ER81 postnatally, we examined ER81 expression in P15 *Ret$^{CFP/+}$; Wnt1^{Cre};TrkA$^{f/f}$* L5 DRGs. Conditional *TrkA* mutants were used here instead of constitutive *TrkA*-nulls because *TrkA*-null mice die at ~P0. We found that 13.5 ± 1.3% of CFP$^+$ neurons express ER81 at this age (Fig. 2*F*). The decrease in the proportion of L5 ER81$^+$ RA mechanoreceptors from P0 to P15 suggests that ER81 is downregulated during postnatal development.

To directly demonstrate that ER81$^+$ DRG neurons innervate Pacinian corpuscles, we crossed a tamoxifen-inducible Cre recombinase driven from the *Er81* locus to a *Tau^{GFP}* conditional reporter allele, and treated pregnant dams with tamoxifen at embryonic day 14.5 (E14.5) and E15.5 to permanently label ER81$^+$

neurons with GFP. At P15, tamoxifen-treated mice displayed GFP expression in a subset of ER81$^+$ DRG neurons (Fig. 2*G*). Notably, a few GFP$^+$ neurons no longer expressed ER81 at P15, consistent with the idea that some DRG neurons may have downregulated *Er81* during development. GFP$^+$ axons innervated layers III-V of the dorsal spinal cord, the target zone of mechanosensory neurons (Fig. 2*H*). Additionally, whole-mount staining of the interosseous membrane showed that a subset of Pacinian corpuscles is innervated by GFP$^+$ axons (Fig. 2*I*). The lack of GFP$^+$ axons innervating all Pacinian corpuscles is likely due to the incomplete recombination efficiencies of the inducible Cre and reporter allele. Collectively, our results indicate that *Er81* is expressed in Pacinian corpuscle-innervating DRG neurons.

Er81 is required in neurons, but not Schwann cells, for Pacinian corpuscle formation during development

To determine in which cell type *Er81* is required for Pacinian corpuscle formation during development, we combined an *Er81* conditional allele with tissue-specific *Cre* lines. We generated conditional mutants with *Nestin^{Cre}*, which drives recombination in Pacinian corpuscle-innervating neurons but not corpuscle Schwann cells (Fig. 3*A*). Anti-ER81 immunostaining was absent from DRG sections of *Nestin^{Cre};Er81$^{f/-}$* mice, demonstrating efficient recombination of the *Er81^f* allele in DRG neurons (Fig. 3*B*,*C*). Serial sections of P7 mutant hindlimbs revealed a complete absence of Pacinian corpuscles around the fibula (Fig. 3*D*,*E*). Therefore, *Er81* is absolutely required in neurons for Pacinian corpuscle formation during development.

Because *Er81* is also expressed in the Pacinian corpuscle inner core Schwann cells, we next asked whether *Er81* in Schwann cells also contributes to Pacinian corpuscle development. We used a *Desert Hedgehog^{Cre}* (*Dhh^{Cre}*) transgenic line, which drives recombination in Pacinian corpuscle Schwann cells but not their innervating neurons (Fig. 3*F*), to ablate *Er81*. At P7, *Dhh^{Cre}*;
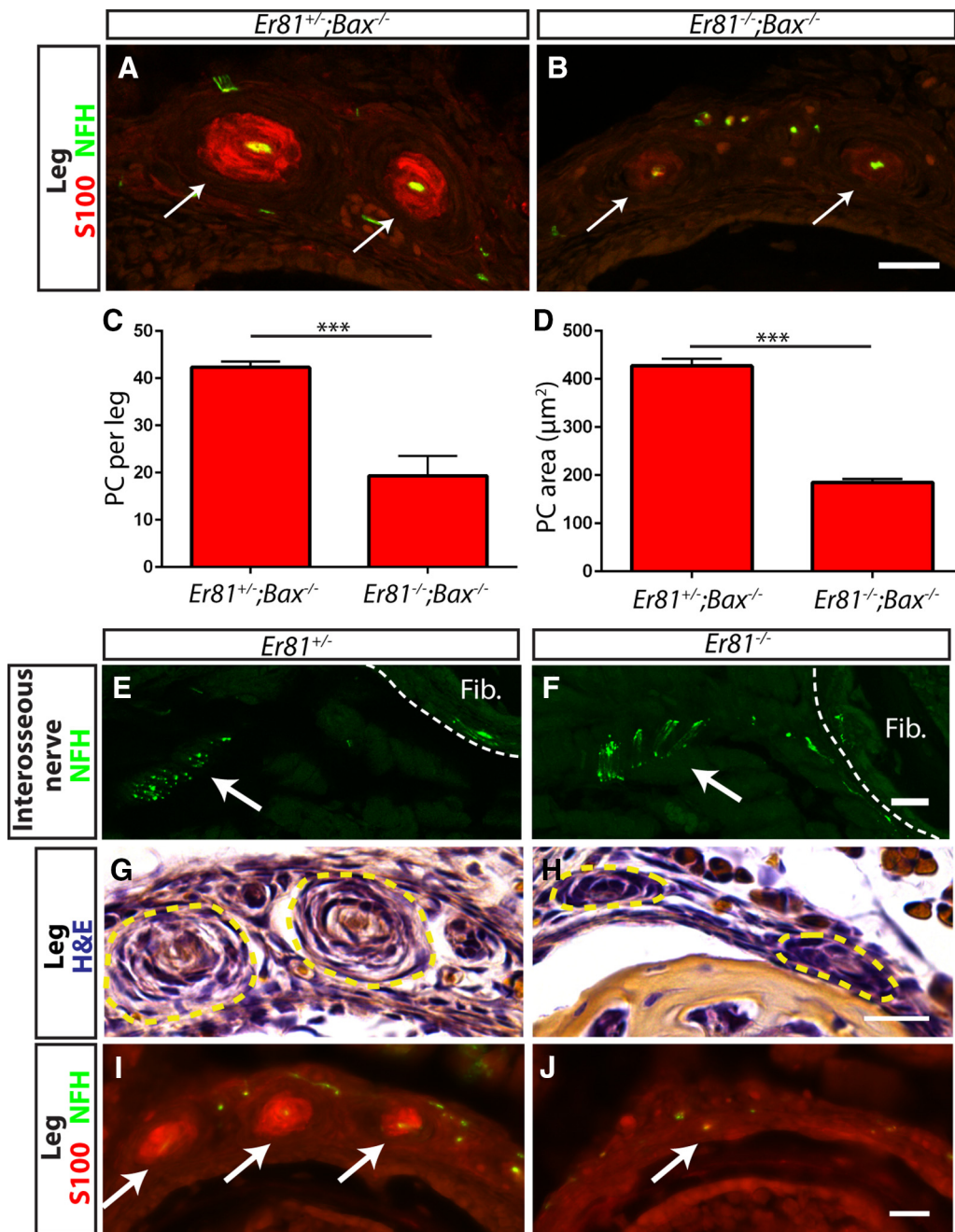
Figure 8. The primary deficit in Pacinian corpuscle formation in *Er81* mutants is deficient axon/Schwann cell communication. *A*, *B*, Anti-S100 (red) and anti-NFH (green) staining of P3 *Er81*$^{+/−}$;*Bax*$^{−/−}$ control (*A*) and *Er81*$^{−/−}$;*Bax*$^{−/−}$ double-mutant (*B*) hindlimb sections. Arrows indicate Pacinian corpuscles. Note the lower expression level of S100 in double-mutant Pacinian corpuscles relative to controls. *C*, Quantification of S100$^+$ Pacinian corpuscles in serial leg sections reveals a significant decrease in the number of corpuscles formed in double mutants relative to controls (42.33 ± 1.20 PC per leg in *Er81*$^{+/−}$;*Bax*$^{−/−}$ controls, 19.33 ± 4.18 PC per leg in *Er81*$^{−/−}$;*Bax*$^{−/−}$ double mutants; p = 0.006). *D*, Quantification of average cross-sectional area of Pacinian corpuscle inner cores reveals a significant decrease in the size of the remaining Pacinian corpuscles in double mutants (427.3 ± 14.7 μm^2 in *Er81*$^{+/−}$;*Bax*$^{−/−}$ controls, 185.3 ± 6.7 μm^2 in *Er81*$^{−/−}$;*Bax*$^{−/−}$ double mutants; p = 0.0001). *E*, *F*, Anti-NFH staining of P2 interosseous nerve shows intact innervation of fibula/interosseous membrane in both *Er81*$^{+/−}$ control (*E*) and *Er81*$^{−/−}$ mutant (*F*) mice. *G*, *H*, H&E staining of P2 hindlimbs shows early Pacinian corpuscles forming in the *Er81*$^{+/−}$ control (*G*). However, Schwann cells form much smaller, rudimentary Pacinian corpuscles in the *Er81*$^{−/−}$ mutant (*H*). *I*, *J*, Anti-S100 (red) and anti-NFH (green) staining shows that mature Schwann cells are present in corpuscles by P2 in the control (*I*) but are absent in the mutant (*J*). N = 3 animals, 6 legs per genotype. Scale bar, 20 μm. ***p < 0.001. Error bars indicate SEM.

Er81$^{f/−}$ mutant mice had significantly fewer and smaller Pacinian corpuscles (Fig. 3G–J). Nevertheless, the phenotype in the Schwann cell-specific mutants is much less severe compared with that of neural-specific mutants. Additionally, the Pacinian corpuscle number in adult *Dhh*^{Cre};*Er81*$^{f/−}$ mutant mice catches up to control levels (Fig. 4E), suggesting that development of Pacinian corpuscles may be delayed when *Er81* is ablated from Schwann cells. Thus, *Er81* in Schwann cells contributes to, but is less critical for, Pacinian corpuscle formation during development.

Er81 in Schwann cells is not required for Pacinian corpuscle maintenance following injury

Although nerve innervation is absolutely required for the developmental formation of Pacinian corpuscles (Zelená, 1980), Pa-

cinian corpuscles can survive for up to 1 year upon denervation/nerve injury in the adult (Zelená, 1982, 1984). The exact mechanisms underlying this nerve-ind-ependent maintenance of adult Pacinian corpuscles are not known. Given the less severe phenotype of $Dhh^{Cre};Er81^{f/-}$ mu-tant mice, we wondered whether neuronal factors may compensate for the loss of $Er81$ in Schwann cells and that its role in Schwann cells would be more obvious upon denervation. Thus, we performed unilateral tibial nerve transection on 2- to 4-month-old $Dhh^{Cre};Er81^{f/-}$ conditional mutants and $Er81^{f/-}$ control mice, and ex-amined Pacinian corpuscles 2 weeks after surgery. We confirmed denervation by the loss of NFH$^+$ fibers in Pacinian corpus-cles (Fig. 4A–D). However, the number of corpuscles in the transected leg was similar to that of the contralateral nonop-erated leg in both the controls and condi-tional mutants (Fig. 4E). This result indicates that $Er81$ is not necessary for the survival of Pacinian corpuscle Schwann cells following denervation.

$Er81$ functions downstream of Ret in Pacinian corpuscle-innervating neurons

The common Pacinian corpuscle pheno-type between Ret and $Er81$ mutants sug-gests that they may act in a common molecular pathway to promote corpuscle formation. If so, reducing the dosage of both genes may augment the effect on corpuscle formation. To test this idea, we examined the number of Pacinian corpuscles per leg in wild-type, $Ret^{+/-}$, $Er81^{+/-}$, and $Ret^{+/-};Er81^{+/-}$ double-heterozygous mice. We found that Ret and $Er81$ single heterozygotes did not have a sig-nificant decrease in the number of Pacinian corpuscles relative to wild-type mice. In contrast, $Ret^{+/-};Er81^{+/-}$ double heterozy-gotes had significantly fewer Pacinian corpuscles per leg than all other genotypes, and this deficit was greater than the additive deficit exhibited by Ret and $Er81$ single mutants (Fig. 5A). This result indicates that Ret and $Er81$ act in common or parallel path-ways for Pacinian corpuscle formation during development.

How could RET and ER81 interact? In proprioceptors, $Er81$ expression is downstream of TrkC/NT3 neurotrophic signaling (Patel et al., 2003). Thus, we hypothesize that an analogous RET/ neurturin signaling pathway may control the expression of $Er81$ in Pacinian corpuscle-innervating RA mechanoreceptors. To test whether Ret regulates the expression of $Er81$, we performed double-fluorescent $in\ situ$ hybridization against CFP (driven from the Ret locus) and $Er81$ in L4/L5 DRG sections. At E13.5, shortly after $Er81$ begins to be expressed in DRG neurons (Arber et al., 2000), we did not observe a change in the proportion of CFP^+ neurons that express $Er81$ in $Ret^{CFP/CFP}$ mutants compared with $Ret^{CFP/+}$ controls (Fig. 5B–D). Therefore, Ret is not required for the initiation of $Er81$ expression in RA mechanoreceptors. We next examined the expression of $Er81$ at E16.5 and found that a smaller proportion of RA mechanoreceptors expressed $Er81$ in Ret mutants compared with controls, suggesting a requirement for RET signaling in maintaining the expression of $Er81$ at later embryonic stages (Fig. 5E–G).
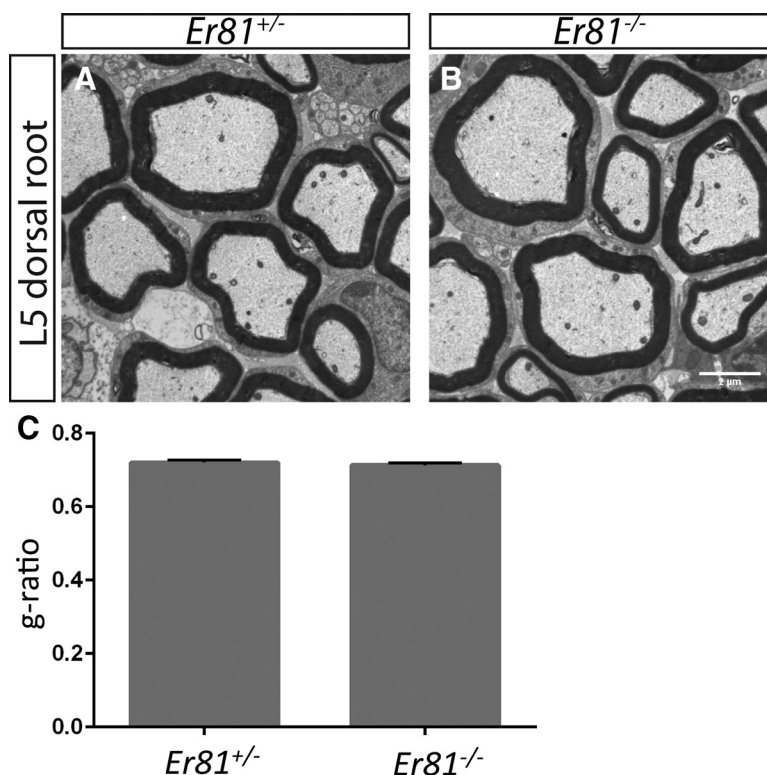
Figure 9. $Er81$ is not required for myelination of peripheral nerves. **A**, **B**, Transmission electron micrographs of L5 dorsal roots from P21 $Er81^{+/-}$ control and $Er81^{-/-}$ mutants reveal no defect in the morphology of the myelin sheath surrounding large-diameter sensory neurons in mutant mice. **C**, The g-ratios of myelinated axonal fibers are similar between $Er81$ mutants and controls (0.720 ± 0.007 in $Er81^{+/-}$ controls, 0.7132 ± 0.006 in $Er81^{-/-}$ mutants; $p = 0.50$). $N = 3$ animals per genotype, >100 fibers per animal. Scale bar, 2 μm.

One potential confound when examining the loss-of-function effects of neurotrophic factor signaling is neuronal death. It re-mains possible that decreases in $Er81$ expression in Ret mutants may be due to the death of specific subsets of RA mechanorecep-tors. To investigate cell-survival independent effects of RET sig-naling on $Er81$ expression, we examined Ret mutants and controls in a Bax-null background, which blocks cell death caused by a lack of neurotrophic support (Patel et al., 2000). We found a significant decrease in the proportion of large-diameter CFP^+ neurons expressing $Er81$ in P0 $Ret^{CFP/CFP};Bax^{-/-}$ double mutants compared with $Ret^{CFP/+};Bax^{-/-}$ controls (Fig. 5H–J). Together, our results indicate that, similar to NT3/TrkC signaling in proprioceptors, RET signaling is required for the maintenance, but not initiation, of $Er81$ expression in RA mechanoreceptors.

Conversely, it is possible that $Er81$ regulates the expression of Ret. To examine this possibility, we performed $Ret/TrkA$ double-fluorescent $in\ situ$ hybridization combined with anti-NFH immunostaining with $Er81^{+/-};Bax^{-/-}$ control and $Er81^{-/-};Bax^{-/-}$ double-null L4/L5 DRG sections (Fig. 6A, B). We used the Bax-null background here as well because loss of DRG neu-rons during the early postnatal period in $Er81$ mutants has pre-viously been reported (Kucera et al., 2002; de Nooij et al., 2013). We quantified the number of $Ret^+/TrkA^-/NFH^+$ RA mechano-receptors per L4/L5 DRG section (Fig. 6C). We found that this number was not significantly different between Bax single-null controls and the $Er81;Bax$ double mutants, suggesting that $Er81$ does not regulate the expression of Ret in RA mechanoreceptors. Collectively, our results place $Er81$ downstream of Ret in the pathway controlling Pacinian corpuscle formation.
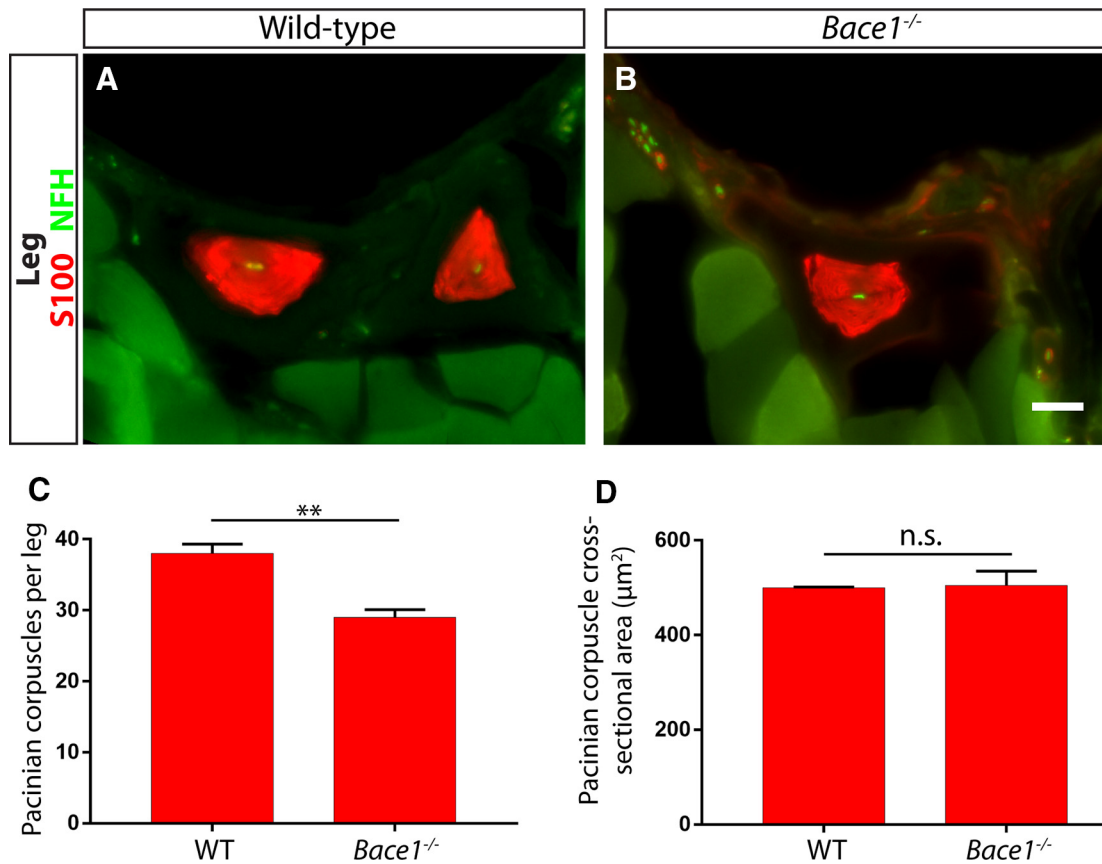
Figure 10. *Bace1* contributes to the development of Pacinian corpuscles. *A*, *B*, Anti-S100 and anti-NFH staining with hindlimb sections of 3- to 4-week-old *Bace1*$^{+/+}$ control (*A*) and *Bace1*$^{-/-}$ mutant (*B*) mice show Pacinian corpuscles around the fibula. *C*, *Bace1*-null mice have significantly fewer S100$^+$ Pacinian corpuscles per leg quantified from serial hindlimb sections compared with wild-type littermate controls (*Bace1*$^{+/+}$ mice have 38 ± 1.31 Pacinian corpuscles per leg, *Bace1*$^{-/-}$ mice have 29 ± 1.10 Pacinian corpuscles per leg; $p = 0.006$). *D*, The size of the remaining Pacinian corpuscles in *Bace1* mutants is not different from those in controls (500.3 ± 1.43 μm^2 cross-sectional area in *Bace1*$^{+/+}$ mice, 505.4 ± 29.82 μm^2 cross-sectional area in *Bace1*$^{-/-}$ mice; $p = 0.87$). $N = 3$ animals, 6 legs per genotype. **$p < 0.01$. Not significant: $p \geq 0.05$. Error bars indicate SEM. Scale bar, 20 μm.

Primary deficits of Pacinian corpuscle development in *Ret* and *Er81* mutants

What are the primary deficits in *Ret* and *Er81* mutants that prevent Pacinian corpuscle formation? To determine whether the *Ret* Pacinian corpuscle phenotype is completely due to the death of Pacinian corpuscle-innervating neurons, we examined *Ret*$^{-/-}$;*Bax*$^{-/-}$ double mutants at P0, the latest time they survive. Because S100 is not yet expressed in immature Schwann cells at P0, we used H&E staining of serial leg sections to visualize rudimentary Pacinian corpuscles. We found rudimentary corpuscles around the fibula of *Ret*$^{CFP/+}$;*Bax*$^{-/-}$ control mice, but not in the *Ret*$^{CFP/CFP}$;*Bax*$^{-/-}$ double mutants (Fig. 7*A*,*B*). In addition, we performed immunostaining against CFP driven from the *Ret* locus to examine the axons of the interosseous nerve innervating Pacinian corpuscles. These axons were found adjacent to the fibula and proximal to the formation of Pacinian corpuscles in the *Ret*$^{CFP/+}$;*Bax*$^{-/-}$ control mice but were absent in the *Ret*$^{CFP/CFP}$;*Bax*$^{-/-}$ double mutants (Fig. 7*C*,*D*). Therefore, Pacinian corpuscle development is not initiated in *Ret* mutants, which is likely due to deficient axon guidance or growth of Pacinian corpuscle-innervating neurons in the absence of RET signaling.

Why do Pacinian corpuscles fail to form in *Er81* mutants? We considered three potential functions of ER81 in neurons to control Pacinian corpuscle development. First, it is possible that Pacinian corpuscle-innervating neurons depend on *Er81* for their survival. Second, similar to *Ret* mutants, Pacinian corpuscle-innervating neurons may not reach their target without *Er81*.

Last, if innervating neurons reach the proper target and Schwann cell precursors are present, a problem in communication between the axon and the Schwann cells could prevent corpuscle formation. To determine whether *Er81* acts to promote neuronal survival to control Pacinian corpuscle formation, we performed anti-S100/anti-NFH immunostaining on *Er81* mutant and control legs in a *Bax*-null background at P3 (Fig. 8*A*,*B*). We found that, although S100$^+$ Pacinian corpuscles were present in the double mutants, they were more than halved in number and size (Fig. 8*C*,*D*). Additionally, the expression level of S100, which is a marker of Schwann cell maturation, was just above the threshold of detection in the double mutants and much weaker compared with that of the *Er81*$^{+/-}$;*Bax*$^{-/-}$ control corpuscles. Therefore, *Er81* mutants have defects in Pacinian corpuscle development other than cell death. In addition, because all corpuscles were innervated by NFH$^+$ axons in controls, *Er81*$^{-/-}$ single-null (Fig. 8*E*,*F*) and *Er81*$^{-/-}$;*Bax*$^{-/-}$ double mutants, our results also suggest that targeting and growth of Pacinian corpuscle axons are largely unaffected in the absence of *Er81*.

If innervating axons reach their target in *Er81* mutants, do they begin the process of generating Pacinian corpuscles? We performed H&E and anti-S100/anti-NFH staining on P2 leg serial sections. In *Er81*$^{+/-}$ controls, we observed well-formed rudimentary corpuscles by H&E staining. In addition, Schwann cells surrounding innervating fibers started to express high levels of S100, a marker of mature Schwann cells. In contrast, *Er81*-nulls had hypotrophic rudimentary Pacinian corpuscles by H&E
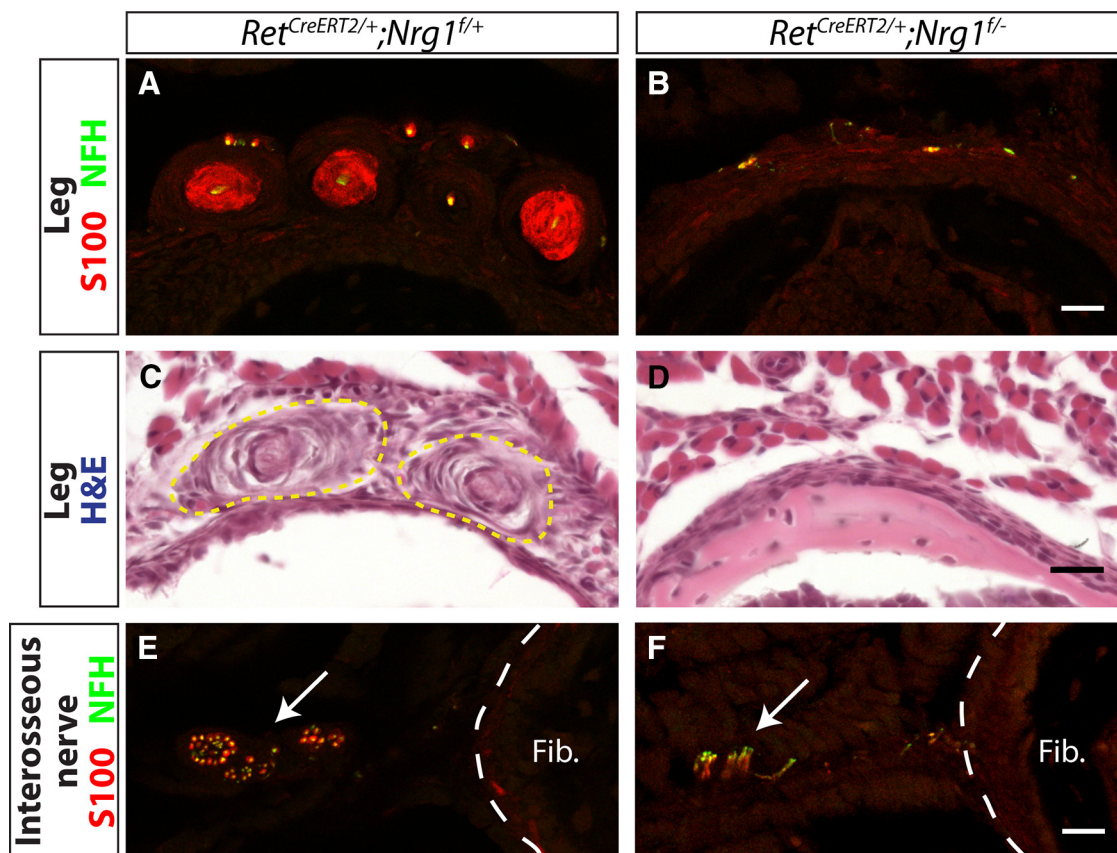
Figure 11. *Nrg1* is required in neurons for Pacinian corpuscle formation. $Ret^{CreERT2/+}$;$Nrg1^{f/+}$ controls and $Ret^{CreERT2/+}$;$Nrg1^{f/-}$ mutants were treated with tamoxifen at E13.5 and E14.5 to ablate *Nrg1* conditional allele in Ret^+ cells, including Pacinian corpuscle-innervating neurons. **A**, **B**, Anti-S100 (red) and anti-NFH (green) staining of P5 hindlimb sections shows a lack of S100$^+$ Pacinian corpuscles in the *Nrg1* conditional mutant mice. **C**, **D**, H&E-stained hindlimb sections show a lack of Pacinian corpuscle-like Schwann cell structures in *Nrg1* conditional mutant mice. **E**, **F**, S100/NFH staining of hindlimb sections proximal to the appearance of Pacinian corpuscles around the fibula shows axons of the interosseous nerve, which innervate Pacinian corpuscles, present in both mutant and control mice. $N = 3$ animals, 6 legs per genotype. Scale bar, 20 μm.

staining, whereas Pacinian corpuscle-like anti-S100 staining was not present around the NFH$^+$ axons innervating the fibula (Fig. 8G–J). This lack of mature Schwann cell phenotype is very similar to that of P3 $Er81^{-/-}$;$Bax^{-/-}$ double-mutant mice. Collectively, our results indicate that *Er81* mutant Pacinian corpuscle-innervating axons reach their target and initiate the development of Pacinian corpuscles. However, Schwann cells of these rudimentary corpuscles do not properly differentiate to form mature Pacinian corpuscles. Given the absolute requirement of *Er81* in neurons to control Pacinian corpuscle development (Fig. 3A–F), these phenotypes suggest that ER81 may play critical roles in innervating axons to communicate with the corpuscle-forming Schwann cells. Our analysis of *Ret* and *Er81* mutant Pacinian corpuscle phenotypes also supports the idea that RET functions upstream of ER81 in this pathway.

Normal myelination in *Er81* mutants
Given the Pacinian corpuscle phenotype of *Er81* mutant mice and *Er81* expression in myelinating Schwann cells (Srinivasan et al., 2007), we asked whether the development of the most prevalent Schwann cell type in the PNS, myelinating Schwann cells, is also affected by the ablation of *Er81*. To answer this question, we performed transmission electron microscopy on L5 dorsal roots of $Er81^{+/-}$ controls and $Er81^{-/-}$ mutants and calculated the g-ratio of individual axonal fibers (Fig. 9). At P21, which is near the maximum lifespan of *Er81* mutants, there was no significant difference in the g-ratio between controls and mutants, and there

were no obvious defects in the morphology of the myelin sheath. Therefore, in contrast to its critical role for Pacinian corpuscle formation, *Er81* does not seem to be required for development of myelinating Schwann cells, proper myelination, or for communication between axons and myelinating Schwann cells.

Nrg1 is required in RA mechanoreceptors for Pacinian corpuscle formation
Because ER81 is a transcription factor, it is unlikely that it acts directly in the communication between axons and nonmyelinating Schwann cells. Which molecules could function downstream of ER81 to mediate this action? Pacinian corpuscle-innervating neurons express NRG1, whereas corpuscle Schwann cells express the NRG1 receptors ERBB2 and ERBB3 (Kopp et al., 1997; González-Martínez et al., 2007). However, it is unclear whether NRG1 signaling contributes to Pacinian corpuscle formation during development.

The juxtamembrane regions of both NRG1-CRD and NRG1-Ig can be cleaved by proteases (Nave and Salzer, 2006; Fleck et al., 2013), which may potentiate the ability of the EGF domain of NRG1 to interact with ERBB receptors. β-secretase 1 is one of the proteases, which cleaves NRG1 and contributes to the promyelinating and promuscle spindle development roles of *Nrg1-CRD* and *Nrg1-Ig*, respectively (Hu et al., 2006; Willem et al., 2006; Cheret et al., 2013). Thus, we first examined the legs of *Bace1*-nulls and littermate controls. We found a significant decrease in the number of Pacinian corpuscles per leg, but no dif-
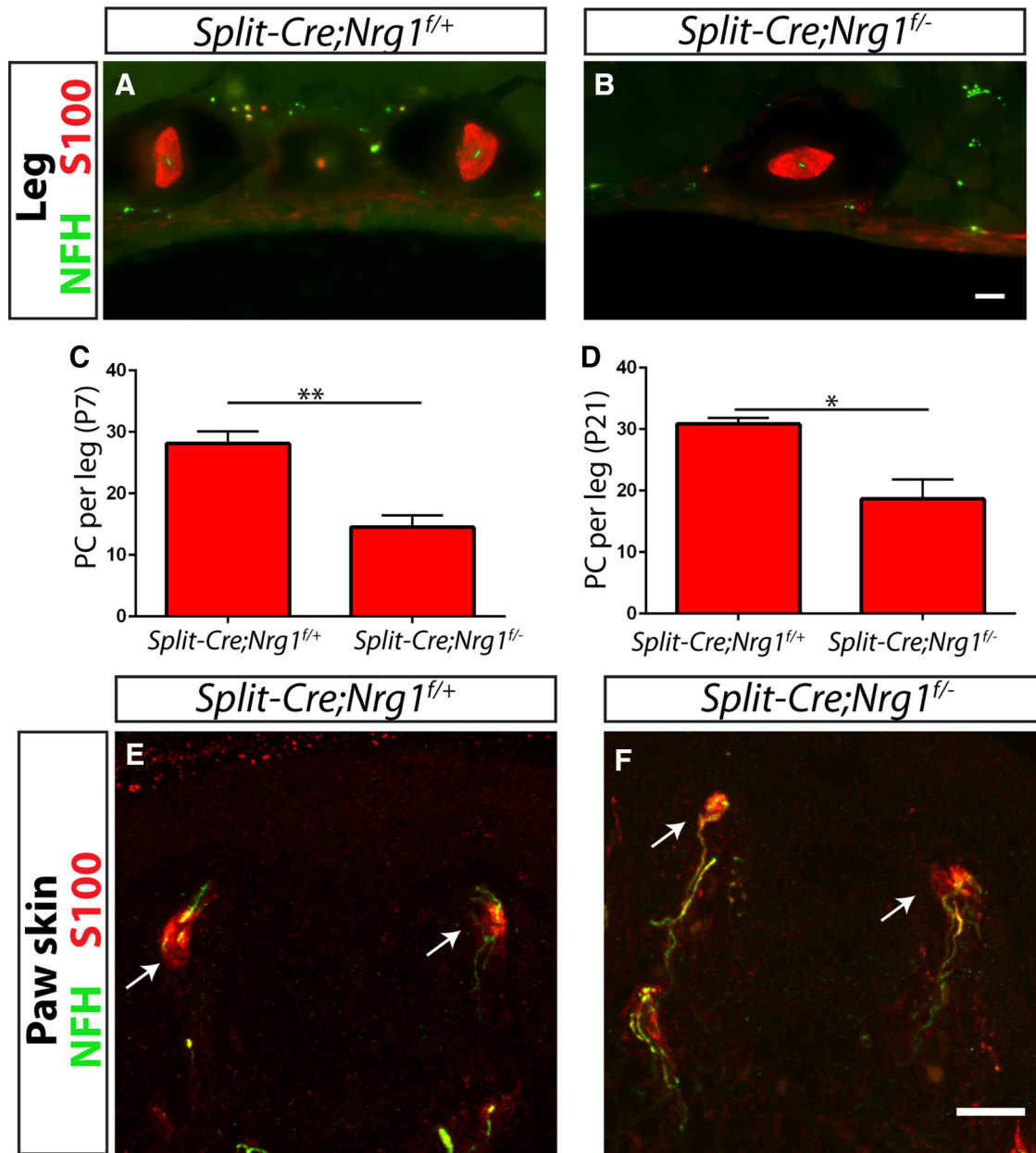
Figure 12. Neuronal *Nrg1* is required for Pacinian, but not Meissner, corpuscle development. *A–D*, *Split^Cre*, which drives recombination in RA mechanoreceptors, produced a partial, but significant, decrease in the number of Pacinian corpuscles per leg when combined with a conditional *Nrg1* allele at both P7 (data not shown; *C*) and P21 (*A*, *B*, *D*) (*Split^Cre*;*Nrg1^{f/+}* controls have 28.13 ± 1.98 corpuscles per leg at P7, *Split^Cre*;*Nrg1^{f/−}* mutants have 14.50 ± 1.92 corpuscles per leg at P7, *p* = 0.002; *Split^Cre*;*Nrg1^{f/+}* controls have 30.83 ± 1.01 corpuscles per leg at P21, *Split^Cre*;*Nrg1^{f/−}* mutants have 18.67 ± 3.18 corpuscles per leg at P21; *p* = 0.02). *E*,*F*, Glabrous skin sections from P21 *Split^Cre*;*Nrg1^{f/+}* controls (*E*) and *Split^Cre*;*Nrg1^{f/−}* mutants (*F*) show no obvious change in the appearance or number of Meissner corpuscles in mutants relative to controls. *N* = 3 animals, 6 legs/glabrous skin samples per genotype. *p < 0.05. **p < 0.01. Error bars indicate SEM. Scale bar, 20 μm.

ference in the average size of individual corpuscles (Fig. 10). A decrease in number, rather than a total absence of corpuscles, is in line with partial reductions in myelination and muscle spindle formation observed in *Bace1* mutants (Hu et al., 2006; Willem et al., 2006; Cheret et al., 2013). This phenotype suggests that *Nrg1* may be required for Pacinian corpuscle formation.

To determine whether neuronal *Nrg1* expression is required for Pacinian corpuscle formation, we ablated *Nrg1* from RA mechanoreceptors by combining a floxed allele of *Nrg1* with a tamoxifen-inducible *Ret^CreERT2* line (Luo et al., 2009). We treated these mice with tamoxifen at E13.5 and E14.5 to avoid early embryonic requirements for *Nrg1* in DRG neuron survival and cardiovascular development. At P5, Pacinian corpuscles and

innervating axons were present in the *Ret^CreERT2*;*Nrg1^{f/+}* control mouse legs, whereas in *Ret^CreERT2*;*Nrg^{f/−}* conditional mutant legs, S100^+ Pacinian corpuscles were completely absent (Fig. 11 *A*, *B*). As expression of molecular markers, such as S100, may be altered in the mutant background, we also confirmed the absence of Pacinian corpuscle structures in conditional mutants by H&E staining (Fig. 11 *C*,*D*). Interestingly, NFH^+ innervating axons persisted, suggesting that Pacinian corpuscle axons reach their target and survive until at least P5 when *Nrg1* expression is ablated (Fig. 11 *E*,*F*). Therefore, similar to *Er81*, *Nrg1* is not required for axon pathfinding of Pacinian corpuscle neurons but likely functions to mediate communication between axons and corpuscle-forming Schwann cells.
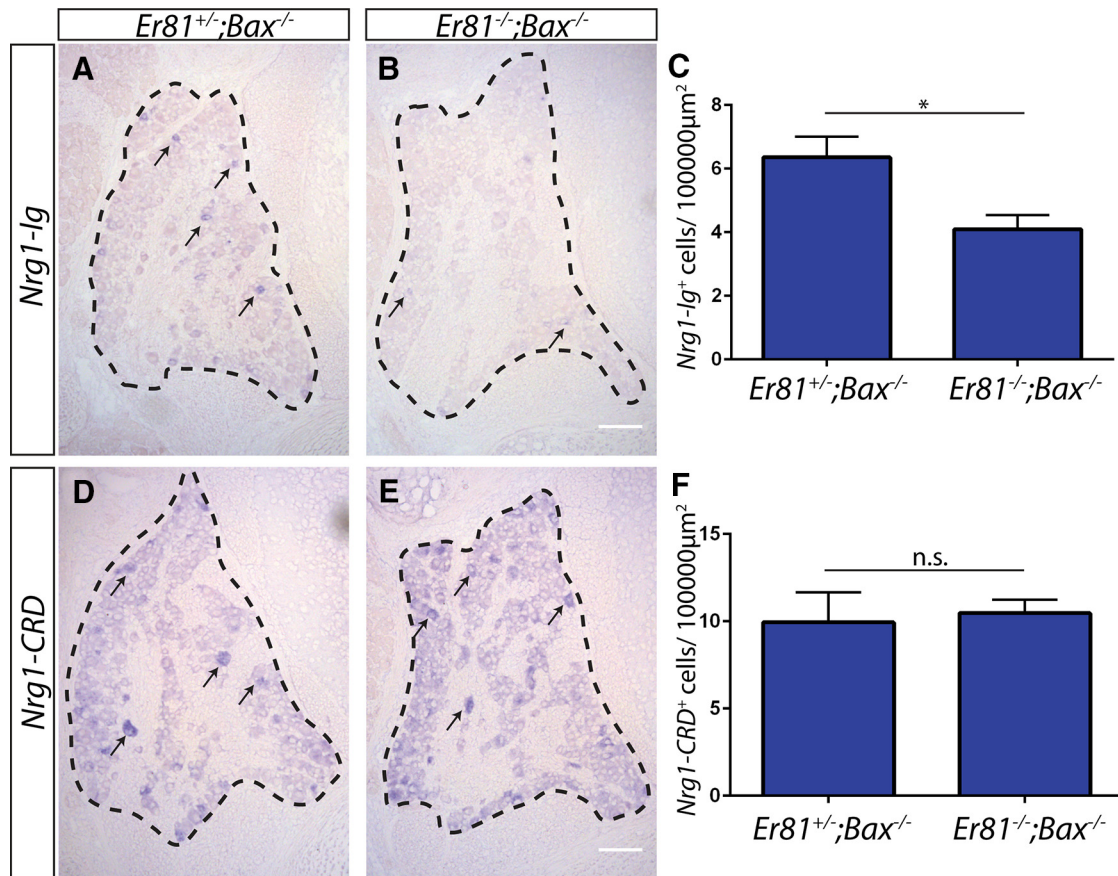
Figure 13. *Er81* regulates the expression of *Nrg1-Ig*, but not *Nrg1-CRD*, in DRG neurons. ***A–C***, Isoform-specific *in situ* hybridization against *Nrg1-Ig* shows a significant decrease in the number of cells expressing *Nrg1-Ig* transcript in L4/L5 DRGs from P3–P5 *Er81* mutants in an apoptosis-deficient background relative to controls (6.36 ± 0.65 *Nrg1-Ig*$^+$ cells per 100,000 μm^2 in *Er81*$^{+/-}$; *Bax*$^{-/-}$ controls, 4.09 ± 0.48 *Nrg1-Ig*$^+$ cells per 100,000 μm^2 in *Er81*$^{+/-}$;*Bax*$^{-/-}$ controls; $p = 0.04$). Some positive cells are indicated by arrows. ***D–F***, Isoform-specific *in situ* hybridization against *Nrg1-CRD* shows no difference in the number of DRG neurons expressing a high level of *Nrg1-CRD* transcript between controls and mutants (9.94 ± 1.76 *Nrg1-CRD*$^+$ cells per 100,000 μm^2 in *Er81*$^{+/-}$;*Bax*$^{-/-}$ controls, 10.47 ± 0.77 *Nrg1-CRD*$^+$ cells per 100,000 μm^2 in *Er81*$^{+/-}$;*Bax*$^{-/-}$ controls; $p = 0.79$). $N = 3$ animals per genotype, ≥8 sections per animal. *$p < 0.05$. Not significant: $p \geq 0.05$. Error bars indicate SEM. Scale bar, 100 μm.

To confirm the neural requirement for *Nrg1* in Pacinian corpuscle formation, we also ablated *Nrg1* from mechanosensory neurons using a *Split*^{Cre} transgenic line, which also drives recombination in RA mechanoreceptors (Rutlin et al., 2014). At P7 and P21, *Split*^{Cre};*Nrg1*$^{f/-}$ conditional mutants have approximately half the number of Pacinian corpuscles of *Split*^{Cre};*Nrg1*$^{f/+}$ control mice (Fig. 12A–D). The incomplete phenotype observed in *Split*^{Cre} conditional mutants may be caused by incomplete ablation of *Nrg1* in mechanosensory neurons due to transient or low-level expression of recombinase, or due to a late ablation of *Nrg1* in some neurons after a critical period. In addition, no obvious defects of Meissner corpuscles were found in P21 *Split*^{Cre}; *Nrg1*$^{f/-}$ conditional mutants (Fig. 12 E,F), suggesting that Meissner corpuscles do not depend on neuronal *Nrg1* for their end organ formation. Thus, *Nrg1* is specifically required in RA mechanoreceptors for Pacinian corpuscle development.

Nrg1-Ig expression is reduced in *Er81*-null DRG neurons
Given the very similar Pacinian corpuscle phenotypes in *Er81*-null and *Nrg1* conditional-null mutants, we asked whether *Er81* regulates the expression of *Nrg1*. We performed isoform-specific *in situ* hybridization for *Nrg1-Ig* and *Nrg1-CRD* transcripts. We found that the expression of *Nrg1-Ig*, but not *Nrg1-CRD*, transcript was reduced in *Er81*;*Bax* double-null L4/L5 DRGs (Fig. 13), suggesting that *Er81* specifically regulates *Nrg1-Ig* expres-

sion. This finding is in agreement with the g-ratio data, which showed no deficits in myelination in *Er81* mutants (Fig. 9). Given the tight correlation between myelination and *Nrg1-CRD* expression (Michailov et al., 2004), it also seems unlikely that *Nrg1-CRD* expression would be reduced in *Er81* mutants.

To further investigate a potential role for NRG1-CRD in Pacinian corpuscle development, we examined Pacinian corpuscle formation in *Nrg1-CRD*$^{+/-}$ heterozygotes and wild-type littermate controls at P21. *Nrg1-CRD*-null mice die around birth and have high levels of somatosensory neuron cell death (Wolpowitz et al., 2000), making the examination of Pacinian corpuscles difficult to interpret. *Nrg1-CRD*$^{+/-}$ heterozygotes survive to adulthood and have a large decrease in the level of myelination of peripheral nerves. If the dose-dependent requirement for NRG1-CRD is similar between myelinating Schwann cells and nonmyelinating Pacinian corpuscle Schwann cells, then *Nrg1-CRD* heterozygotes may display some deficits in the number or size of Pacinian corpuscles. However, we found that the size and number of Pacinian corpuscles were not significantly different between *Nrg1-CRD*$^{+/-}$ and wild-type mice (Fig. 14). Therefore, unlike myelinating Schwann cells, Pacinian corpuscle Schwann cells do not require high levels of *Nrg1-CRD* expression for their development. In combination with the *Nrg1* mutant phenotype and *Nrg1* isoform-specific *in situ* data from *Er81* mutant mice, this result suggests that ER81 in Pacinian corpuscle-innervating
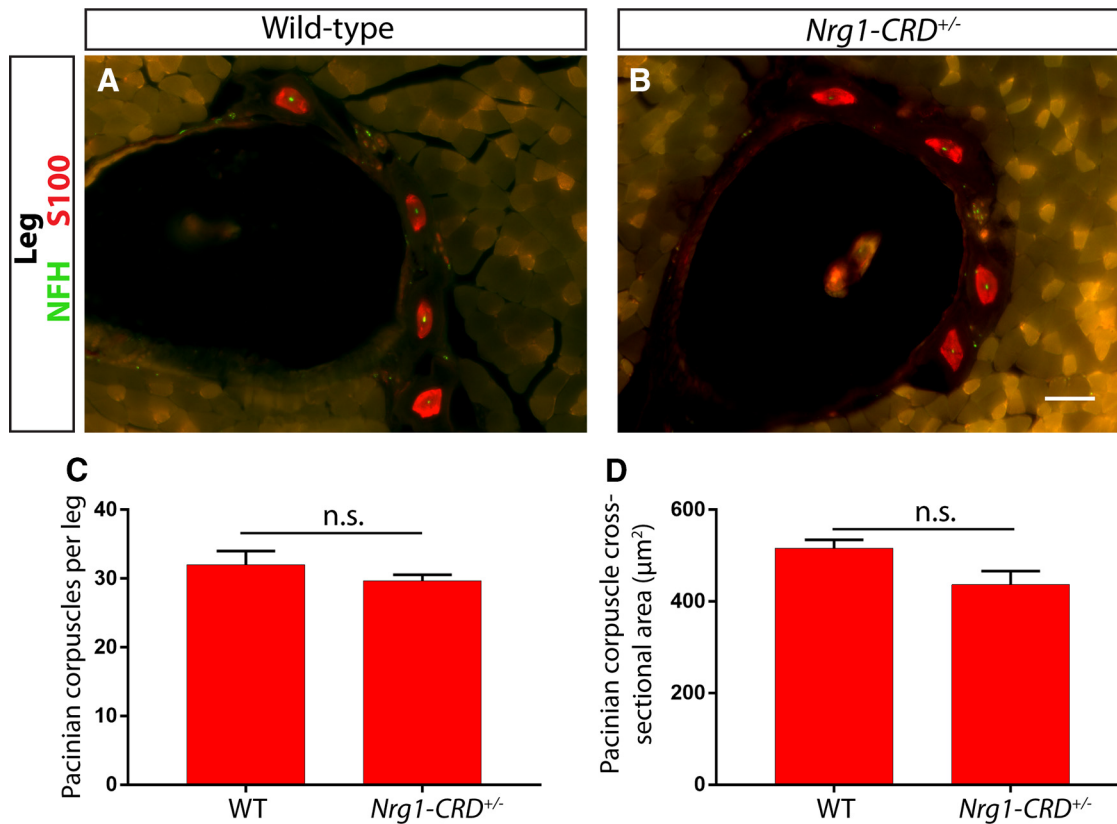
Figure 14. A high level of NRG1-CRD is not required for Pacinian corpuscle development. *A*, *B*, Anti-S100 (red) and anti-NFH (green) staining with hindlimb sections of P21 *Nrg1-CRD*$^{+/+}$ (*A*) and *Nrg1-CRD*$^{+/-}$ (*B*) mice show Pacinian corpuscles around fibula. *C*, *D*, The number of Pacinian corpuscles per leg (*C*) and average size (*D*) of Pacinian corpuscles is similar between *Nrg1-CRD* heterozygotes and wild-type littermate controls (*Nrg1-CRD*$^{+/+}$ mice have 32 ± 2 Pacinian corpuscles per leg, *Nrg1-CRD*$^{+/-}$ mice have 29.67 ± 0.88 Pacinian corpuscles per leg; $p = 0.35$; 516.5 ± 18.33 μm^2 cross-sectional area in *Nrg1-CRD*$^{+/+}$ mice, 436.6 ± 29.57 μm^2 cross-sectional area in *Nrg1-CRD*$^{+/-}$ mice; $p = 0.08$). $N = 3$ animals, 6 legs per genotype. Not significant: $p \geq 0.05$. Error bars indicate SEM. Scale bar, 50 μm.

neurons regulates expression of *Nrg1*, likely *Nrg1-Ig*, to communicate with nonmyelinating Schwann cells and thus promote Pacinian corpuscle formation.

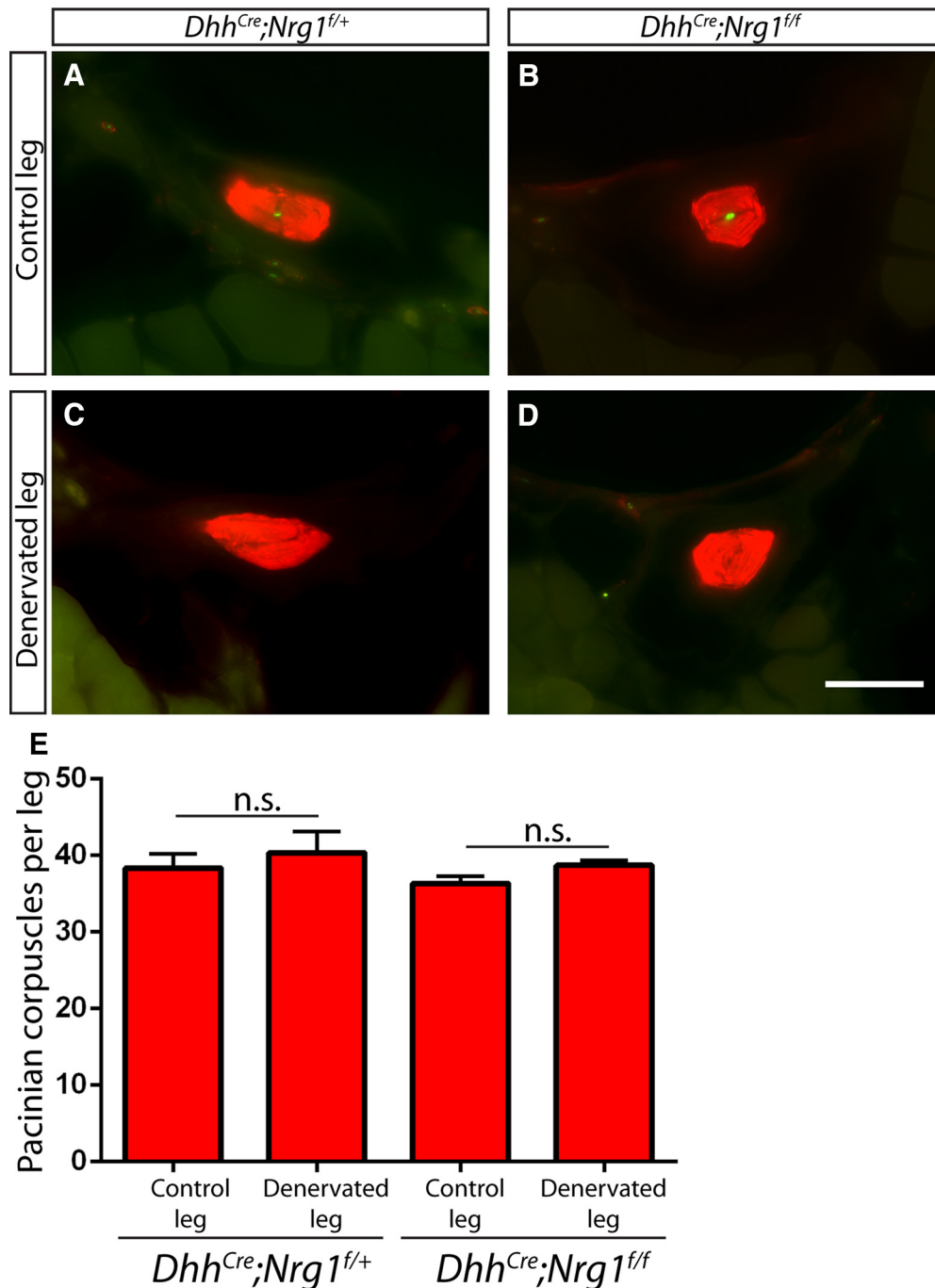### *Nrg1* in Schwann cells is not required for Pacinian corpuscle maintenance following injury

Last, although *Nrg1* is not normally expressed at detectable levels in Schwann cells, it has been reported that myelinating Schwann cells begin to express *Nrg1* following nerve injury, and that autocrine NRG1 signaling allows for the redifferentiation of Schwann cells following denervation (Carroll et al., 1997; Stassart et al., 2013). This Schwann cell-derived NRG1 signaling is critical for nerve regeneration and remyelination. Thus, we investigated whether a similar mechanism exists in Pacinian corpuscle nonmyelinating Schwann cells upon denervation/nerve injury. We generated Schwann cell-specific *Dhh*^{Cre};*Nrg1*$^{f/f}$ mutants and observed no obvious deficits in Pacinian corpuscle number or morphology at P7 (data not shown). We performed unilateral tibial nerve transection on adult controls and conditional mutants and confirmed denervation by a lack of NFH$^+$ innervating fibers. We observed no decrease in the number of corpuscles per leg in the denervated leg relative to the contralateral leg even 3 months after surgery (Fig. 15). Our results suggest that the cellular mechanisms supporting regeneration upon nerve injury also differ between myelinating and nonmyelinating Schwann cells.

Discussion

Nonmyelinating Schwann cells are an important component of the PNS, but how they communicate with neurons has remained largely unclear. In this study, we have used mouse Pacinian corpuscles as a model to investigate axonal interactions with non-myelinating Schwann cells and identified a signaling pathway that specifically functions in this process.

Previous studies have shown that the receptor tyrosine kinase *Ret* and the ETS transcription factor *Er81* both are required for the development of Pacinian corpuscles (Sedý et al., 2006; Luo et al., 2009). However, the mechanisms underlying ER81 function in this regard are unclear. Here, we have shown that *Er81* is expressed and required in Pacinian corpuscle-innervating neurons (Fig. 3), *Er81* and *Ret* genetically interact (Fig. 5) to control Pacinian corpuscle development, and *Er81* expression in RA mechanoreceptors is partly controlled by *Ret* (Fig. 5). One minor difference between our results and the previous study (Sedý et al., 2006) is that we found Pacinian corpuscle afferents and nascent Pacinian corpuscles are present at P2 in *Er81* mutants, whereas the previous study suggested that they are not present in mutants from E16.5 to P1. This discrepancy is likely due to the different methods used for detecting Pacinian corpuscles and their afferents between the previous study and our own, or differences in the genetic backgrounds of the mice that may affect the timing or severity of the phenotypes. In addition, we found that *Nrg1*, a major mediator of axon-Schwann cell communication, is also required in RA mechanoreceptors for Pacinian corpuscle formation, and that ER81 may control the expression of *Nrg1-Ig* to promote Pacinian corpuscle formation (Fig. 13). Thus, our results establish a RET-ER81-NRG1 pathway in neurons to promote Pacinian corpuscle end organ formation (Fig. 16).

Figure 15. Schwann cell-derived *Nrg1* is not required for maintenance of Pacinian corpuscle following denervation. *A–D*, Two- to 4-month-old *Dhh^{Cre};Nrg1^{f/+}* control (*A, C*) and *Dhh^{Cre};Nrg1^{f/f}* conditional mutants (*B, D*) underwent unilateral tibial nerve transection and recovered for 3 months before tissue processing. NFH $^+$ (green) innervating fibers were present in the S100 $^+$ (red) corpuscle inner cores of cross-sections of nonsurgical limbs (*A, B*) but were absent from corpuscle inner cores of denervated limbs (*C, D*). *E*, Quantification of S100 $^+$ Pacinian corpuscles in serial hindlimb sections in *Dhh^{Cre};Nrg1^{f/f}* controls (38.33 $\pm$ 1.86 corpuscles per nonsurgical limb, 40.33 $\pm$ 2.73 corpuscles per denervated limb; $p = 0.58$) and *Dhh^{Cre};Nrg1^{f/f}* conditional mutants (36.33 $\pm$ 0.88 corpuscles per nonsurgical limb, 38.67 $\pm$ 0.67 corpuscles per denervated limb; $p = 0.10$) shows that loss of *Nrg1* in Schwann cells does not cause a deficit in the maintenance of Pacinian corpuscles following denervating injury. $N = 3$ legs per condition. Not significant: $p \geq 0.05$. Error bars indicate SEM. Scale bar, 50 μm.

Our results also suggest that neurons may use different molecules, such as different isoforms of NRG1, to communicate with myelinating versus nonmyelinating Schwann cells. It is remarkable that Pacinian corpuscles are completely absent in *Er81* mutant mice due to a deficit of axon-Schwann cell communication while myelination seems normal (Figs. 8, 9). This phenotype is consistent with the fact that expression of *Nrg1-CRD*, the NRG1 isoform-specific for myelination thickness, is normal in *Er81* mutants and that there is no Pacinian corpuscle phenotype in *Nrg1-CRD* heterozygous mice.

However, it remains an open question whether *Nrg1-Ig* is the key or only isoform required for Pacinian corpuscle development. It was mentioned that Pacinian corpuscles are present in *Nrg1-Ig* conditional mutants (Cheret et al., 2013), but the data were not shown and the age of examination was not described. Therefore, more careful analysis is needed for *Nrg1-Ig* conditional mutants. Alternatively, it is possible that multiple isoforms of NRG1 are sufficient to drive Pacinian corpuscle formation, and eliminating individual isoforms will not produce a serious deficit in corpuscle development.
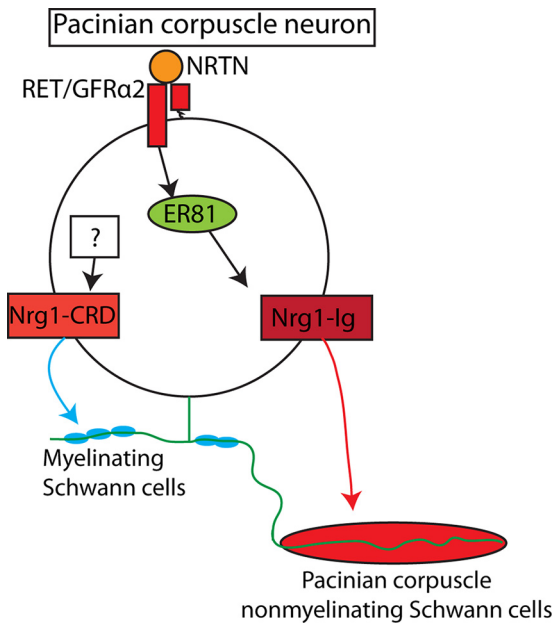
Figure 16. The roles of RET signaling, ER81, and NRG1 in Pacinian corpuscle development. In Pacinian corpuscle-innervating neurons, the expression of ER81 lies downstream of RET/GFRα2/NRTN signaling. ER81 regulates the expression of some *Nrg1* isoforms, including *Nrg1-Ig*, which is required for communicating with corpuscle-forming Schwann cells and thus the formation of Pacinian corpuscle end organs. Another isoform of NRG1, NRG1-CRD, is critical for interactions with myelinating Schwann cells (Michailov et al., 2004), but the upstream transcriptional regulators remain unknown.

Moreover, although the signaling pathways in Schwann cells activated by NRG1 have been extensively studied (Newbern and Birchmeier, 2010; Salzer, 2015), how the isoform-specific expression, as well as expression level, of *Nrg1* is regulated in neurons is completely unknown. Although *Er81* and *Nrg1* mutant mice display very similar muscle spindle phenotypes (Arber et al., 2000; Hippenmeyer et al., 2002), they have not been previously linked in the same pathway. The similar Pacinian corpuscle phenotype of *Er81* and *Nrg1* mutant mice and our isoform-specific *Nrg1 in situ* data (Figs. 11, 13) strongly suggest that ER81 may specifically regulate the expression of *Nrg1-Ig*, but not *Nrg1-CRD*, which provides the first potential transcriptional regulator for *Nrg1* as far as we know. Further studies to determine how ER81 and other factors control the expression level of *Nrg1* in different cell types may assist in understanding not only axon-Schwann cell interactions in the PNS, but also etiology of diseases, such as schizophrenia, in which disruption of *Nrg1* expression has been implicated (Mei and Nave, 2014).

Neurotrophic factor signaling, ER81, and NRG1 in somatosensory neurons

Our findings also raise interesting parallels with proprioceptors, another major class of somatosensory neurons. *Er81* is required in proprioceptors to establish appropriate connections with motor neuron targets in the spinal cord (Arber et al., 2000). Similar to the observed *Er81*-null Pacinian corpuscle phenotype, the initial proprioceptive projections to the periphery are normal in *Er81* mutants, but the morphology and specification of proprioceptive end organs go awry in the early postnatal period (Arber et al., 2000; Kucera et al., 2002). In addition, proprioceptors are highly dependent on TrkC/NT3 signaling for their survival and development (Klein et al., 1994; Tessarollo et al., 1994), and TrkC/NT3 signaling is necessary and sufficient to drive ER81

expression in proprioceptors (Patel et al., 2003). Pacinian corpuscle-innervating neurons express the neurotrophic factor receptor RET, and RET signaling components are required for the formation of Pacinian corpuscles. In *Ret* mutants, we found that the expression of *Er81* is significantly reduced in RA mechanoreceptors. Therefore, these two distinct classes of neuron use distinct neurotrophic signaling pathways to converge on a similar cellular output of ER81 expression. Notably, the *Er81*-null proprioceptive and Pacinian corpuscle phenotypes are less severe than the $NT3^{-/-};Bax^{-/-}$ (Patel et al., 2003) and $Ret^{-/-}; Bax^{-/-}$ phenotypes (Fig. 7), suggesting that neurotrophic signaling engages additional mediators other than *Er81* in both cell types. Last, both proprioceptors and Pacinian corpuscles have similar reliance on *Nrg1* for the development of their respective end organs. In neural-specific *Nrg1* mutants, early stages of muscle spindle induction do not occur (Hippenmeyer et al., 2002). The enriched expression of *Nrg1-Ig* in proprioceptive neurons and examination of isoform-specific *Nrg1* mutants suggest that *Nrg1-Ig*, but not *Nrg1-CRD*, is required for muscle spindle development (Hippenmeyer et al., 2002; Cheret et al., 2013). Here, we have shown that Pacinian corpuscles do not form when *Nrg1* is ablated from RA mechanoreceptors and *Nrg1-CRD* may not to be required in this process as well. Taken our data and previous publications into consideration, it seems possible that *Er81* specifically controls the expression of *Nrg1-Ig* and/or other isoforms in both proprioceptors and Pacinian corpuscle-innervating neurons to control proper end organ development of both types.

In addition to RET, Pacinian corpuscle afferents express additional receptor tyrosine kinases, such as TrkC (Sedý et al., 2004), although there is only a small decrease in the number of Pacinian corpuscles in *TrkC* mutants (Sedý et al., 2004; de Carlos et al., 2006). Nevertheless, as both RET and TrkC promote *Er81* and *Nrg1* expression (Hippenmeyer et al., 2002), it is possible that RET and TrkC signaling may activate common downstream pathways in DRG neurons to promote Pacinian corpuscle development.

Last, both Pacinian and Meissner corpuscles are formed by RA mechanosensory axons and nonmyelinating Schwann cells. Both types of neurons develop from the early RET$^+$ DRG neurons and display rapidly adapting and low-threshold mechanosensitivity. However, Pacinian corpuscles have large multilayer corpuscles, whereas Meissner corpuscles have small incomplete corpuscles. This difference is very important as it not only gives rise to different end organ morphologies but contributes to their physiological properties and optimal vibration detection frequencies (Loewenstein and Skalak, 1966). Given the known correlation between *Nrg1* expression level and myelination thickness, it is interesting to speculate that the ER81-NRG1 pathway we found here is a key molecular mechanism to differentiate Pacinian corpuscles from Meissner corpuscles. Future gain-of-function experiments to overexpress *Er81* or *Nrg1* in Meissner corpuscle-innervating neurons would help to test this idea.

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