Ret Signaling Is Required for Tooth Pulp Innervation during Organogenesis

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Abstract

During organogenesis, the timing and patterning of dental pulp innervation require both chemoattractive and chemorepellent cues for precise spatiotemporal regulation. Our understanding of the signaling mechanisms that regulate tooth innervation during development, as well as the basic biology of these sensory neurons, remains rudimentary. In this study, we analyzed the expression and function of glial cell line–derived neurotrophic factor (GDNF) and its receptor tyrosine kinase, Ret, in the regulation of innervation of the mouse tooth pulp by dental pulpal afferent (DPA) neurons of the trigeminal ganglion (TG). Using reporter mouse models, we demonstrate that Ret is highly expressed by a subpopulation of DPA neurons projecting to the tooth pulp at both postnatal day 7 (P7) and in the adult. In the adult tooth, GDNF is highly expressed by many cell types throughout the dental pulp. Using a ubiquitous tamoxifen (TMX)–inducible Cre (*UBC-Cre/ER^{T2}*) line crossed to Ret conditional knockout mice (*Ret^{fl/fl}*), Ret was deleted immediately prior to tooth innervation, and the neural projections into P7 molars were analyzed. TMX treatment was efficient in ablating >95% of Ret protein. We observed that *UBC-Cre/ER^{T2}; Ret^{fl/fl}* mice had a significant reduction in the total number of neurites present within the pulp at P7, with a significant accumulation of aberrant fibers in the dental follicle and periodontium. In agreement with these findings, inhibition of Ret signaling through in vivo administration of a highly specific pharmacologic inhibitor (INM-PPI) of Ret also caused a substantial reduction in pulpal innervation. Taken together, these findings indicate that Ret signaling regulates the timing and patterning of tooth innervation by dental primary afferent neurons of the TG during organogenesis and provide a rationale to explore whether alterations in the GDNF-Ret pathway contribute to pathophysiological conditions in the adult dentition.

Keywords: GDNF, neurotrophic factor, neurotrophin, nociceptor, trigeminal, sensory

Introduction

The establishment of dental pulp innervation during organogenesis is a highly regulated process, involving both chemoattractive and chemorepellent cues to coordinate innervation from sensory nerves emanating from the trigeminal ganglion (TG). This process is critically important for the health of the organism, as dental pulpal afferents (DPAs) serve a vital role in protecting the dentition from injury and infection. The time course of dental pulp innervation is well characterized in the mouse mandibular molar, making it an ideal model system for studying developmental mechanisms regulating dental pulp innervation (Luukko et al. 2005; Fried et al. 2007). In the mouse, the earliest trigeminal axons reach the tooth germ of the mandibular first molar at embryonic day 13 (E13) (Mohamed and Atkinson 1983), yet axons do not enter the pulp until the final crown shape has been achieved and mineralization begins, at approximately postnatal days 4 to 5 (P4–P5). In this manner, axon pathfinding and pulpal innervation patterning is tightly coupled with tooth formation.

To achieve this tight spatiotemporal regulation of innervation, it has been proposed that chemorepulsive cues are produced in the developing tooth germ embryonically, thereby

inhibiting axonal projections from entering. Postnatally, these repulsive cues are downregulated, followed by the coincident onset of expression of chemoattractive cues, thereby promoting tooth innervation (Luukko et al. 2005; Luukko and Kettunen 2014). While many mediators have been proposed to be involved, Semaphorin 3A (Sema3A) is the best-characterized regulator of pulp innervation. Sema3A is a secreted axon guidance molecule that mediates axon repulsion and growth cone collapse in the peripheral nervous system, exerting its effects through a Neuropilin-Plexin receptor complex (Takahashi et al. 1999). In the developing tooth germ, *Sema3a* is expressed from E10 to E18, becoming more restricted in its expression postnatally (Kettunen et al. 2005). The Sema3A

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coreceptor *Neuropilin-1* is coincidentally expressed by trigeminal neurons and their projections (Gu et al. 2003; Lillesaar and Fried 2004). In *Sema3a*^{-/-} mice, ectopic innervation occurs prematurely by P0, thus leading to abnormal innervation patterns (Moe et al. 2012).

The innervation of the developing tooth pulp is also regulated by the presence of chemoattractant cues. Nerve growth factor (NGF) is highly expressed during and after the period coinciding with the onset of tooth innervation, while nociceptive neurons within the trigeminal ganglion express the high-affinity NGF receptor TrkA (Qian and Naftel 1996; Luukko, Arumae, et al. 1997; Huang et al. 1999). Pulpal afferents in the first mandibular molar (MM1) and incisors of *trkA*^{-/-} mice were markedly reduced (Matsuo et al. 2001), supporting the notion that NGF promotes innervation. However, the interpretation of these data is conflicted by the early trophic dependence of TG neurons on NGF/TrkA signaling before tooth innervation, and thus an early survival deficit would appear to manifest as a deficit in pulpal innervation.

The glial cell line–derived neurotrophic factor (GDNF) family ligands (GFLs) are a family of neurotrophic factors critical for the survival, differentiation, and axon growth of various peripheral neuron populations. The GFLs consist of 4 homologous growth factors: GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN). Together, they exert their functions by first binding to glycerophosphatidylinositol (GPI)–linked coreceptors known as GDNF family receptor- α s (GFR α s), which then engage the receptor tyrosine kinase Ret (Airaksinen and Saarma 2002). Interestingly, the GDNF receptors GFR α 1 and Ret have been found to be highly expressed by trigeminal ganglion neurons beginning as early as E11 (Luukko, Suvanto, et al. 1997), while GDNF is expressed in embryonic and neonatal rat and mouse mandibular molars (Nosrat et al. 1998). Although these findings are highly suggestive of a role for GDNF/Ret signaling in promoting dental pulp innervation, the early perinatal death of *Gdnf*^{-/-} and *Ret*^{-/-} mice has obstructed the subsequent exploration of this hypothesis. In this study, we employed an inducible knockout and pharmacogenetic approach to explore the role of Ret signaling in the development of tooth innervation. We found that trigeminal pulpal afferents express Ret during the time period coinciding with the onset of tooth innervation. Furthermore, we demonstrate that disruption of Ret signaling immediately preceding dental pulp innervation does not alter TG neuron survival but significantly reduces dental pulp afferents at P7. Collectively, these data provide evidence that developmental Ret signaling promotes the proper establishment of tooth innervation, demonstrating a new molecular mediator in the axon pathfinding of trigeminal dental neurons during tooth organogenesis.

Materials and Methods

Detailed experimental materials and methods are provided in the Appendix.

Results

Small- and Large-Diameter Trigeminal Neurons Are Lost between E12.5 and E18.5 in Ret Knockout Mice

Ret is required for the development of numerous dorsal root ganglion (DRG) sensory neuron populations, including rapidly adapting mechanoreceptors (Luo et al. 2009; Lallemand and Ernfors 2012; Kupari and Airaksinen 2014), a subset of CGRP⁻/IB4-binding nociceptors that mediate responses to inflammatory pain and noxious mechanical stimuli (Golden et al. 2010; Franck et al. 2011), and a subset of nociceptors involved in mediating responses to itch (Stantcheva et al. 2016). Despite these findings, and despite several studies indicating important developmental, molecular, and functional differences that exist between the dorsal root and trigeminal ganglia (TGs; Lopes et al. 2017; Nguyen et al. 2017), the role of Ret in TG sensory neuron development remains poorly understood (Naveilhan et al. 1998). To this end, we first sought to determine whether genetic deletion of *Ret* impairs TG sensory neuron survival using *Ret*^{-/-} mice, which remain viable until immediately after birth (Schuchardt et al. 1994). Therefore, we analyzed total cell numbers in *Ret*^{+/+} and *Ret*^{-/-} mice at E12.5 and E18.5. Interestingly, while no difference was observed between genotypes at the earlier timepoint, we observed a drastic reduction in the total number of TG neurons by E18.5 ($41,510 \pm 2,878$ vs. $27,182 \pm 2,381$ neurons; $P = 0.0099$; Fig. 1A). Given that distinct sensory neuron populations can be broadly separated based on the size of their soma (Lallemand and Ernfors 2012), we next analyzed which populations were lost in *Ret*^{-/-} mice at E18.5. Interestingly, we observed that *Ret*^{-/-} mice had significant losses of small-diameter ($\leq 27 \mu\text{m}$) and large-diameter ($\geq 35 \mu\text{m}$) TG neurons (small: 38.82% reduction; $18,073 \pm 1,611$ vs. $11,057 \pm 1,551$ neurons; large: 50.78% reduction; $6,138 \pm 1,007$ vs. $3,021 \pm 276.4$ neurons), while medium-diameter neurons were unaffected (Fig. 1B).

Neurotrophic Factor Expression in Small-, Medium-, and Large-Diameter Trigeminal Sensory Neurons

In the DRG, small-diameter sensory neurons represent a population of unmyelinated polymodal nociceptors that differentiate to form at least 2 distinct subpopulations (Lallemand and Ernfors 2012). Among these is a peptidergic population, characterized by its expression of the calcitonin gene-related peptide (CGRP), with a developmental dependence on NGF/TrkA signaling. During the first postnatal week of life, this population gives rise to a CGRP-negative nonpeptidergic population, characterized by its lack of neuropeptide production and its developmental dependence on GFL/Ret signaling (Golden et al. 2010; Chen et al. 2017). These neurons are functionally distinct (Lallemand and Ernfors 2012), and peptidergic and nonpeptidergic nerve fibers have both been shown to be

present within the adult tooth pulp (Chung et al. 2012). However, whether the peptidergic and nonpeptidergic neurons of the TG share the same neurotrophic dependence as neurons in the DRG is unknown. To this end, we characterized the expression of Ret and TrkA in small-diameter nociceptors using *Ret-Cre/ER^{T2}; Rosa26^{LSL-TdTomato/+}* mice labeled with TMX during the first postnatal week of life (Appendix Fig. S1), which corresponds to the natural time period of tooth innervation. When analyzing small-diameter TG neurons, we observed that Ret (labeled with anti-RFP; green) and TrkA (red) were expressed predominantly in a mutually exclusive pattern (Fig. 1C, D). We also observed a small population of doubly positive neurons (Fig. 1C, D), possibly owing to the ongoing differentiation of peptidergic to nonpeptidergic phenotype.

Previous studies characterizing the DPAs present in the adult tooth have indicated that most neurons are medium to large in diameter (Pan et al. 2003). Thus, we next characterized the relative expression of Ret and TrkA in medium-diameter (27 to 35 μ m) and large-diameter (≥ 35 μ m) TG neurons at P7. Interestingly, while medium-diameter neurons had nearly equal proportions of Ret⁺/TrkA⁻ and Ret⁻/TrkA⁺ neurons and a large proportion of doubly positive Ret⁺/TrkA⁺ neurons, large-diameter neurons were overwhelmingly Ret⁺/TrkA⁻ (Fig. 1C, D).

Ret Is Expressed by Trigeminal Pulpal Afferents during the Developmental Window Coinciding with Tooth Innervation

Given our findings indicating that Ret is expressed in numerous populations of TG sensory neurons, and building upon previous studies demonstrating that GDNF and NRTN are present within the developing tooth germ at a time period coinciding with tooth innervation (Luukko, Arumae, et al. 1997; Luukko, Suvanto, et al. 1997; Nosrat et al. 1998), we sought to determine whether Ret is expressed in early pulpal afferents immediately following tooth innervation. To answer this question, we analyzed the tooth pulp of TMX-labeled *Ret-Cre/ER^{T2}; Rosa26^{LSL-TdTomato/+}* mice at P7. Interestingly, we observed extensive labeling of Ret⁺ fibers via RFP immunostaining, which were primarily localized to the core of the coronal pulp, with no observed labeling of fibers approaching the subodontoblastic layers or beyond (Fig. 2). In addition, we performed immunostaining for Ret (RFP), neurofilament (NF; labels all nerve fibers), and myelin basic protein (MBP; labels

Figure 1. Characterization of embryonic trigeminal ganglion (TG) deficits in Ret knockout mice and postnatal neurotrophic factor receptor expression. (A) TGs were isolated from embryonic day 12.5 (E12.5) and E18.5 *Ret^{+/+}* and *Ret^{-/-}* mice. Tuj1 immunostaining was performed and the total number of TG neurons was assessed by counting Tuj1⁺ neurons with intact nuclei. No difference was observed between genotypes at E12.5 ($n = 5$ *Ret^{+/+}* and $n = 3$ *Ret^{-/-}* mice). At E18.5, *Ret^{-/-}* mice had significantly fewer TG neurons ($P = 0.0099$; $n = 5$ per genotype). (B) The number of small-, medium-, and large-diameter TG neurons was quantified at E18.5. *Ret^{-/-}* mice had significantly fewer small- and large-diameter TG neurons (small: $P = 0.0139$; large: $P = 0.0175$; 2-tailed t test; $n = 5$ per genotype). Data represent the mean $\pm$ SEM. (C, D) *Ret-Cre/ER^{T2}; Rosa26^{LSL-TdTomato/+}* mice were given tamoxifen (TMX) as described in Appendix Figure 1. (C) Postnatal day 7 (P7) TGs were serially sectioned and stained for RFP (green) and TrkA (red). Small-, medium-, and large-diameter neurons were classified as Ret⁺, TrkA⁺, or Ret⁺/TrkA⁺. (D) Quantifications of C. TrkA expression is highest in small-diameter neurons, while Ret expression is highest in large-diameter neurons.

myelinating Schwann cells and myelination) and observed that the majority of Ret⁺ fibers overlapped with NF and did not overlap MBP (Appendix Fig. S2), indicating these fibers are likely unmyelinated C fibers.

Ret Is Required for Target Innervation of the Tooth Pulp

To test the hypothesis that Ret is required for tooth innervation, we crossed *Ret^{fx/fx}* mice with *UBC-Cre/ER^{T2}* mice, a TMX-inducible Cre line driven by the ubiquitously expressed ubiquitin C promoter, thereby allowing for the temporal control of Ret deletion to avoid the postnatal lethality of *Ret^{-/-}* mice. Importantly, we have previously used this genetic approach to delete Ret in other systems, which was highly efficacious (Donnelly et al. 2018). TMX was directly administered to neonatal *Ret^{fx/fx}* or *Ret^{fx/fx}; UBC-Cre/ER^{T2}* pups from P0 to P3, and TGs and mandibles were collected at P7. To determine the efficacy of TMX deletion, TGs were then lysed and detergent extracted, followed by immunoblotting for Ret and actin. We found that TMX induction in this manner led to a near-complete loss of Ret in *Ret^{fx/fx}; UBC-Cre/ER^{T2}* mice compared to *Ret^{fx/fx}* littermate controls (Fig. 3A, B). To test the hypothesis that Ret is required for tooth innervation, we next analyzed

Figure 2. Ret is expressed in trigeminal pulpal afferents during the period of tooth innervation. Mandibles from postnatal day 7 (P7) *Ret-Cre/ER^{T2}; Rosa26^{LSL-TdTomato/+}* mice were serially sectioned and immunostained for Sytox (green) and RFP (labeling Ret; blue), with the merged image displayed in the right-most panel. The dashed box is magnified in the lower 2 panels. We observed a substantial number of Ret⁺ nerve fibers within the pulp, as well as extensive labeling in the nerve immediately below the tooth germ. No labeling was observed in nonneuronal cells. Experiments were performed 3 separate times with similar results. Additional immunostaining for Ret (RFP) in conjunction with Schwann cell/myelination markers is displayed in Appendix Figure S2.

neurites within the mouse mandibular first molar (MM1) using the approach described in Appendix Figure S3, with immunostaining performed for NF to stain all nerve fibers. Interestingly, we observed a striking reduction of the overall number of neurites present within the tooth pulp of *Ret^{fx/fx}; UBC-Cre/ER^{T2}* mice, with a coincident thickening of the nerve branch immediately below the tooth germ, when compared to *Ret^{fx/fx}* controls (Fig. 3C, D).

Pharmacogenetic Blockade of Ret Signaling Is Sufficient to Disrupt Target Innervation of the Tooth Pulp

To further confirm the finding that Ret is required for target innervation of the developing tooth, we crossed *Ret^{fx/fx}* mice with germline knockout mice to generate *Ret^{fx/-}* mice, or *Ret^{fx/+}* mice as a control, as heterozygous mice are indistinguishable from wild-type littermates (Schuchardt et al. 1994). The Ret conditional knockout mice were created to harbor a single-nucleotide missense mutation (V805A) in the tyrosine kinase domain, which is functionally silent but renders Ret susceptible to a highly selective kinase inhibitor, 1NM-PP1 (Chen et al. 2005). Because Ret signaling in vivo functions via a positive feedback loop, blocking Ret kinase activity greatly reduces Ret expression (Luo et al. 2007), allowing us to use this system as a second means of reducing Ret levels during the developmental time corresponding with tooth innervation. 1NM-PP1 (16.6

ng/g body weight) was directly administered to neonatal *Ret^{fx/+}* or *Ret^{fx/-}* pups from P0 to P6, and TGs and mandibles were again collected at P7. To determine the efficacy of 1NM-PP1, TGs were lysed, detergent extracted, and immunoblotted for Ret and actin. We observed a highly significant reduction of Ret in *Ret^{fx/-}* mice compared to *Ret^{fx/+}* littermate controls (Fig. 4A, B), thus substantiating this approach to block Ret signaling. Interestingly, when analyzing the neurites observed throughout the entire MM1, we observed a significant reduction in the total number of neurites present within the tooth pulp of *Ret^{fx/-}* mice compared to *Ret^{fx/+}* controls (Fig. 4C, D), with a similar proportion of neurite loss that was observed in *Ret^{fx/fx}; UBC-Cre/ER^{T2}* mice (43.9% vs. 40.7% reduction). Based on these findings, we conclude that Ret signaling mediates tooth innervation during the first postnatal week of life.

Total TG Neuron Number Is Not Impaired by Loss of Ret Function

Based on the reduction of pulpal afferents following disruption of Ret signaling, we hypothesized that these effects are due to a local loss of chemoattraction and that TG DPA neuron numbers were not changed. To test this hypothesis, we performed total TG cell counts on both experimental models used in Figures 3 and 4. Interestingly, we observed no statistically significant reduction in TG neurons (Appendix Fig. S4). While DPA neurons account for only a subpopulation of TG neurons, these data support the notion that Ret is locally required for innervation of the tooth germ and that the observed effects are not secondary to a loss of TG neurons.

The Adult Tooth Pulp Expresses GDNF and Ret

To determine whether Ret⁺ fibers exist in the adult tooth, we again used *Ret-Cre/ER^{T2}; Rosa26^{LSL-TdTomato}* mice. Adult mice were administered TMX for 5 consecutive days. Mandibles were isolated, prepared as described in Appendix Figure S3, and hemisections were immunostained for NF (green), vimentin (blue; a marker of mesenchymal cells), and RFP (red, indicating Ret⁺ cells). As a control, the MM1 of *Rosa26^{LSL-TdTomato}* wild-type littermates were analyzed, which confirmed the specificity of RFP labeling (Fig. 5A). Interestingly, when analyzing the MM1 of *Ret-Cre/ER^{T2}; Rosa26^{LSL-TdTomato}* mice, we observed extensive labeling of Ret⁺ nerve fibers, including within the dentinal tubules, as well as in many nonneuronal cells throughout the pulp, many of which coexpressed the mesenchymal marker vimentin. Labeling of nonneuronal cells was

especially strong in the pulp horns, where a large number of elongated Ret⁺/vimentin⁺ cells were observed. Some of this labeling may be due to Ret⁺ fibers making extensive contact with vimentin⁺ cells, as neurofilament also appeared on nonneuronal cells. Ret⁺ nerve fibers were also found traversing through the accessory canals and within the periodontal ligament (Fig. 5B). We next analyzed MM1 taken from *Gdnf-Cre/ER^{T2}*; *Rosa26^{LSL-tdTomato}* mice, administered TMX, and immunostained as described in Figure 5A. While no GDNF⁺ nerve fibers were observed in any area analyzed, we observed GDNF⁺ cells that were primarily located within the cell-rich zone throughout the radicular and coronal pulp, with especially prevalent labeling within the pulp horns (Fig. 5C). GDNF⁺ cells were not labeled with MBP, indicating that myelinating Schwann cells do not express GDNF (Appendix Fig. S5). Collectively, these data indicate that GDNF-Ret signaling may serve additional homeostatic functions in the adult tooth.

Discussion

A Model of Tooth Pulp Innervation during Organogenesis

Previous studies detailing the spatiotemporal dynamics of pulpal innervation (Luukko et al. 2005), coupled with studies demonstrating the temporally dynamic upregulation of GDNF and NRTN during the period of tooth innervation (Luukko, Suvanto, et al. 1997), suggested a role for GFL-Ret signaling as a mediator of chemoattraction of dental pulpal afferents during tooth organogenesis. Our data directly test this hypothesis, demonstrating that a loss of Ret signaling specifically during the early postnatal period is sufficient to act as a partial blockade to tooth innervation. It remains unclear, however, to what extent the residual fibers remain due to the presence of compensatory mechanisms, a lack of axon repulsion, or the presence of other growth factors, such as NGF or brain-derived neurotrophic factor (BDNF), both of which have been demonstrated to be expressed in the postnatal tooth germ during the period of tooth innervation (Nosrat et al. 1997). Further, given the presence of the other GFLs in the developing tooth pulp (Nosrat et al. 1998), it remains unclear which individual GFLs are mediating these effects. Given the complexity of target innervation of any tissue, especially one as richly

Figure 3. Ret is required for target innervation of the tooth pulp. (A) *Ret^{flox/flox}* mice with or without *UBC-Cre/ER^{T2}* were administered tamoxifen (TMX) as indicated. Trigeminal ganglia (TGs) were then harvested, lysed, and subjected to immunoblotting for Ret and actin. (B) Quantification of Ret (normalized to actin) indicated *Ret^{flox/flox}*; *UBC-Cre/ER^{T2}* (*Ret*-cKO) mice had significantly reduced levels of Ret ($P = 0.0002$; $n = 6$ per group; 2-tailed t test). (C) Representative images from *Ret^{flox/flox}* and *Ret^{flox/flox}*; *UBC-Cre/ER^{T2}* mice mandibular first molar (MM1), immunostained with neurofilament (green). Fewer innervating fibers were observed in *Ret*-cKO pulp, with a large cluster of nerve fibers below the tooth germ (red arrow head). (D) Higher magnification insets of the white-dashed boxes in panel B (right: *Ret^{flox/flox}*; left: *Ret^{flox/flox}*; *UBC-Cre/ER^{T2}*). (E) The total number of neurites (>5 mm of continuous length) was quantified. *Ret*-cKO mice had a 39% reduction in neurites within the pulp ($P < 0.001$; 2-tailed t test). No change was observed in the overall number of TG neurons (Appendix Fig. 3). Red arrowhead indicates an aberrant nerve fiber bundle underneath the developing pulp.

innervated as the tooth pulp, it is likely that many signaling mechanisms operate in tandem to govern pulpal innervation.

Distinct Actions for GFL-Ret Signaling Regulating Survival, Target Innervation, and Homeostasis?

Our findings that *Ret^{-/-}* TGs are indistinguishable from *Ret^{+/+}* mice at E12.5 but have substantially fewer neurons by E18.5 suggests that Ret is required for the survival and/or proliferation of TG neurons during embryonic development (Fig. 1). However, our data also demonstrate that loss of Ret during the

Figure 4. Pharmacogenetic inhibition of Ret kinase activity is sufficient to phenocopy genetic deletion of Ret. **(A)** $Ret^{+/+}$ or $Ret^{-/-}$ mice were administered the highly selective Ret kinase inhibitor 1NM-PP1 (16.67 ng/g body weight) via intraperitoneal injection from P0 to P6 and also maintained in the drinking water (1 mM) until tissues were harvested at postnatal day 7 (P7). Trigeminal ganglia (TGs) were isolated, lysed, and detergent extracted, followed by immunoblotting for Ret and actin (as a loading control) to verify knockdown. **(B)** Quantification of Ret (normalized to actin) indicated a highly statistically significant reduction in the degree of Ret expression in $Ret^{-/-}$ mice compared to $Ret^{+/+}$ controls ($P = 0.0002$; 2-tailed t test; $n = 5$ per genotype). **(C)** Mandibles were dissected from animals described in A, serially sectioned, and immunostained for neurofilament. **(D)** The total number of neurites was quantified as in Figure 3. 1NM-PP1-treated $Ret^{-/-}$ mice had significantly fewer pulpal afferents compared to $Ret^{+/+}$ controls ($P = 0.0261$; 2-tailed t test). Data represent the mean $\pm$ SEM.

first week of postnatal life impairs target innervation of the tooth pulp without affecting neuron survival (Figs. 3 and 4). There are, therefore, at least 2 distinct functions for Ret in TG neuron development: an early function in survival and/or proliferation dependence of at least 2 broad populations of neurons and a later chemoattractive function that regulates target innervation of DPAs and likely other craniofacial targets. Given our data indicating that Ret is highly expressed within many pulpal afferents, while GDNF is produced by nonneural cells within the pulp (Fig. 5), it is also tempting to propose a third, as of now unexplored role for GDNF-Ret signaling in the maintenance of dental pulpal afferents. Interestingly, Kvinnsland et al. (2004) demonstrated that approximately

one-third of dental pulpal afferents were able to retrogradely transport iodinated GDNF delivered intrapulpally; most were large in diameter and negative for the nociceptive marker TrpV2, indicating that GDNF-Ret signaling may support a distinct subpopulation of pulpal afferents in the adult. Future studies aimed at understanding the function of GDNF in the adult under both physiological and pathophysiological conditions are warranted and may yield insights that are relevant to regenerative endodontic therapies.

The Dental Pulp as a Complex Neurosensory Target Organ

While it has been classically proposed that the tooth pulp is afferently served by a homogeneously nociceptive population of TG neurons, studies using retrograde tracing of TG pulpal afferents revealed a surprising degree of heterogeneity in both size and morphological properties of these neurons, leading to the proposal that TG dental neurons are composed of several populations, with cellular characteristics of both nociceptive and mechanosensitive neurons (Pan et al. 2003; Kim et al. 2011; Vang et al. 2012). While it has long been known that dental pulpal afferents express neuropeptides such as CGRP and substance-P (SP), Chung et al. (2012) demonstrated that nonpeptidergic (CGRP⁻) IB4⁺/MrgprD⁺ dental afferents are present within the dental pulp, suggesting the existence of at least 2 distinct nociceptive populations. Furthermore, a third population of medium- to large-diameter neurons that express the mechanosensitive ion channel Piezo2 and the mechanosensory marker NF200 was recently demonstrated to be present in the pulp (Won

et al. 2017). Many of the neurons in the TG that retrogradely transport iodinated GDNF are large-diameter, TRPV2⁺ neurons, suggesting that they may be mechanosensitive in function (Kvinnsland et al. 2004). In this study, we discovered that Ret⁺ fibers only make up a subset of the overall population of dental pulpal afferents in the adult. As a future direction, it will be important to use genetic models allowing for the ablation or silencing of these specific subpopulations of trigeminal dental neurons using the molecular handles that have been identified. Perhaps only then will we fully understand the cellular basis that underlies the ability of dental pulp to respond to heat, cold, inflammatory, noxious mechanical, and chemical stimuli with exquisite sensitivity.

Figure 5. Expression of Ret and glial cell line–derived neurotrophic factor (GDNF) in adult teeth. **(A)** Mandibles from adult *Rosa26^{LSL-TdTomato/+}* or **(B)** *Ret-Cre/ER^{T2}; Rosa26^{LSL-TdTomato/+}* mice administered tamoxifen (TMX) for 5 consecutive days were serially sectioned and immunostained for neurofilament (green), vimentin (blue), and RFP (labeling Ret; red), followed by confocal imaging of the first mouse mandibular molar (MM1). The merged image is displayed in the right-most panel. **(A)** RFP immunostaining of wild-type *Rosa26^{LSL-TdTomato/+}* demonstrates the specificity of the RFP antibody. **(B)** Extensive labeling of Ret⁺ nerve fibers was observed throughout the radicular and coronal tooth pulp. The dashed box indicates the magnified area displayed in the second row of panels. Ret⁺ nerve fibers can be observed extending into dentinal tubules in the magnified panel. Nonneuronal Ret⁺ cells are also found in the adjacent keratinized gingiva, as well as throughout the pulp chamber, with particularly extensive labeling observed in the coronal pulp horns. **(C)** *Gdnf-Cre/ER^{T2}; Rosa26^{LSL-TdTomato/+}* mice were administered TMX and mandibles stained as described in panel A. No labeling of nerve fibers was observed. Similar to Ret, GDNF⁺ cells were distributed throughout the radicular and coronal pulp, with particularly intense labeling of GDNF⁺ cells within the cell-rich zone of the pulp horns. For all experiments, at least 4 mice were analyzed per genotype with similar results. Scale bars indicate 500 μ m.

Author Contributions

C.R. Donnelly, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; A.A. Shah, E.B. Suh, contributed to data acquisition and analysis, critically revised the manuscript; B.A. Pierchala, contributed to conception, design, data analysis and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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