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ORIGINAL ARTICLE

Expression patterns of Sema3F, PlexinA4, -A3, Neuropilin1 and -2 in the postnatal mouse molar suggest roles in tooth innervation and organogenesis

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Abstract

Objective. Semaphorins form a family of axon wiring molecules but still little is known about their role in tooth formation. A class 3 semaphorin, Semaphorin3F (Sema3F), besides acting as a chemorepellant for different types of axons, controls a variety of non-neuronal developmental processes. ***Materials and methods.*** Cellular mRNA expression patterns of Sema3F as well as neuropilin 1 (Npn1), neuropilin 2 (Npn2), plexinA3 and plexinA4 receptors were analyzed by sectional *in situ* hybridization in the mouse molar tooth during postnatal days 0–7. The expression of the receptors was studied in PN5 trigeminal ganglia. ***Results.*** Sema3F, Npn1, -2 and plexinA4 exhibited distinct, spatiotemporally changing expression patterns, whereas plexinA3 was not observed in the tooth germs. Besides being expressed in the base of the dental mesenchyme *Sema3F,* like *plexinA4, Npn1 and -2,* was present in the ameloblast cell lineage. *Npn1* and *Npn2* were additionally seen in the pulp horns and endothelial cells and like PlexinA4 in the developing alveolar bone. *Npn1, plexinA3* and *-A4* were observed in trigeminal ganglion neurons. ***Conclusions.*** Sema3F may act as a tooth target-derived axonal chemorepellant controlling establishment of the tooth nerve supply. Furthermore, Sema3F, like Npn1, -2 and plexinA4 may serve non-neuronal functions by controlling the development of the ameloblast cell lineage. Moreover, Npn1 and Npn2 may regulate dental vasculogenesis and, together with PlexinA4, alveolar bone formation. Further analyses such as investigation of transgenic mouse models will be required to elucidate *in vivo* signaling functions of Sema3F and the receptors in odontogenesis.

Key Words: *tooth, odontogenesis, axon guidance,* in situ *hybridization*

Introduction

Like many mammalian organs, tooth develops from epithelial and mesenchymal tissue components. Morphologically the formation of the tooth starts as a thickening of the dental epithelium. Subsequently the dental epithelium forms a bud around which the dental mesenchyme condenses. During the following cap and bell stages, the epithelium of the molar tooth undergoes tooth-specific folding morphogenesis. The final shape of the molar tooth crown is determined by the crown mineralization when secretion of the enamel and dentin by amelo- and odontoblasts occurs. Thereafter, the roots start to develop at specific sites in the future enamel–cement interface and consequently tooth eruption into the oral cavity begins.

Sequential as well as reciprocal interactions mediated by different families of signaling molecules regulate the tooth formation and its sensory innervation [1–3]. The class 3 semaphorins (Sema3A-3G), a semaphorin sub-family, is a family of proteins implicated in a variety of cellular developmental processes of various organs [3–5]. With the exception of Sema3E, they require both neuropilin and plexin transmembrane receptors [6–11]. Neuropilin acts as a binding unit, whereas the plexin receptor subunit plays an essential role in transduction of semaphorin signals to the cytoplasm [6–10]. Based on their structure plexins are sub-classified into four groups: A, B, C and D [9], of which plexinAs (plexinA1-A4) and D1 act as receptors for class 3 semaphorins (Sema3s) [6–11]. So far two neuropilins, Npn1 and -2, have been characterized [7].

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During development, class 3 semaphorins and their receptors are expressed in both neuronal and non-neuronal tissues in a spatio-temporally regulated manner [4,5], suggesting a broad range of functions [4,5]. In developing mouse tooth, Sema3s and their receptors Npn1, -2 show developmentally regulated mRNA expression in both epithelial and mesenchymal components [12–14] and additionally *plexinA1* has been reported to be present in embryonic cap and bell stage tooth germ [15], suggesting multiple functions for Sema3 signaling in tooth formation. Reduction of *Sema3A* and *-3F* expression after birth was reported using RT-PCR on dental papilla and pulp samples [13]. Npn1 [6,16,17], Npn2 [18], plexinA3 [15,19] and plexinA4 [15,20] transcripts have been reported in a broad range of peripheral nervous system (PNS) neurons in the mouse including trigeminal, dorsal root and superior cervical ganglion during embryogenesis. Nerve fibers in the peridental mesenchyme of the embryonic tooth bud were demonstrated to be immunopositive to Npn1, but not to Npn2 [13]. Furthermore, although no immunoreactivity for Npn1 or Npn2 in dental pulp nerve fibers at PN5 were reported; Npn2 was observed in the inferior alveolar nerve [13].

Besides acting as a chemorepellant to sympathetic, motoric trochlear, olfactory bulp and hippocampal axons [19,21–23], Sema3F repels endothelial cells of the vascular system [24]. In addition, Sema3F has been demonstrated to have various other non-neuronal functions in cell attachment, migration, apoptosis, proliferation and cytoskeletal organization [5,25]. Furthermore, it has been reported to be involved in lung formation, tumor metastasis and function as a tumor suppressor [5,25]. Sema3F mediates its cellular effects by interacting preferentially with the Npn2/plexinA3-receptor complex [26]. This preferential functional association is not absolute, as Sema3F may also signal partly through Npn1/plexinA4 [20,22,23,26,27]. Moreover, in guidance of trunk neural crest migration Sema3F/Npn2 appears to be sufficient [28].

Despite the fact that much information is available about class 3 semaphorins and their receptors, however, still little is known about their cellular expression patterns and potential functional contribution to a developing postnatal tooth germ and its supporting tissues. Therefore, in the present study, we have investigated the cellular mRNA expression patterns of Sema3F and its known binding receptors Npn1 and Npn2 as well as the signal transducing receptors plexin3A and plexinA4 in the mouse molar tooth germ during postnatal crown morphogenesis and early dentin and enamel formation stages, when the initial ingrowth of the sensory trigeminal and sympathetic nerve fibers into dental pulp occurs. We used sectional radioactive *in situ* hybridization to examine the mRNA expression patterns from postnatal day 0 (PN0) to postnatal day 7 (PN7). Moreover, to address putative roles of Sema3F in dental axon guidance, we investigated mRNA expression of Npn1, Npn2, plexinA3 and plexinA4 receptors in the trigeminal ganglion at PN5.

Results

Expression of Sema3F, neuropilins and plexinA3 and -A4 in the developing postnatal molar tooth germ

At birth (PN0), the mouse mandibular first molar tooth germ is at the bell stage but crown morphogenesis is not ready yet, dentin and enamel deposition has not yet started and nerve fibers have not penetrated into the dental papilla. *In situ* hybridization analysis on tissue sections revealed that *Sema3F* was largely restricted to the epithelial tissue component of the tooth, namely the stellate reticulum, stratum intermedium, cervical loops and in the outer enamel epithelium (Figures 1A1 and B1). In addition, *Sema3F* hybridization signal was seen in restricted sites of the inner enamel epithelium (arrow in Figure 1B1). Similar epithelial expression of *Sema3F* continued in the PN3 stage when dentin formation was evident. *Sema3F* expression was also discerned in mesenchymal cells of the dental papilla (PN0) and pulp (PN3) between the cervical loops (Figures 1B1 and B2). As reported earlier oral and skin epithelium as well as hair follicles displayed some Sema3F transcripts [5] (Figures 1B1–B3, and not shown). *Sema3F* mRNAs were not seen in the dental mesenchyme after PN3, but expression continued in the epithelial stellate reticulum and stratum intermedium cells (Figures 1B3, B4, D2 and D4).

Whereas *plexinA3* expression was not observed in the tooth germs, plexinA4 mRNAs were prominently expressed in the epithelial tissue component throughout the studied stages. At PN0, transcripts were seen in some parts of the inner enamel epithelium including the efa (enamel-free area) preameloblasts (efap) next to the epithelial TEK [29] (Figures 1C1 and D1). In addition, some plexinA4 transcripts were detected in the dental papilla between the cervical loops (thin arrow in Figure 1C1) as well as in the developing alveolar bone (arrowheads in Figure 1C1). At PN3, PN5 and PN7, *plexinA4* expression appeared in the preameloblasts, secreting and efa ameloblasts (Figures 1C2–C4 and D1). However, no hybridization signal was present in the Hertwig's epithelial root sheath (Figures 1C3, C4 and D3). The post-secretory ameloblasts did not show a *plexinA4* signal after the termination of enamel secretion (Figure 1C4).

Npn1 expression was seen in the epithelial stellate reticulum and mesenchymal dental follicle cells surrounding the tooth germ at newborn stage (Figure 2B1). In addition, the dental papilla including the pulp horns and preodontoblasts, but excluding the region close to cervical loops, displayed transcripts (Figure 2B1). Furthermore, some localized *Npn1*

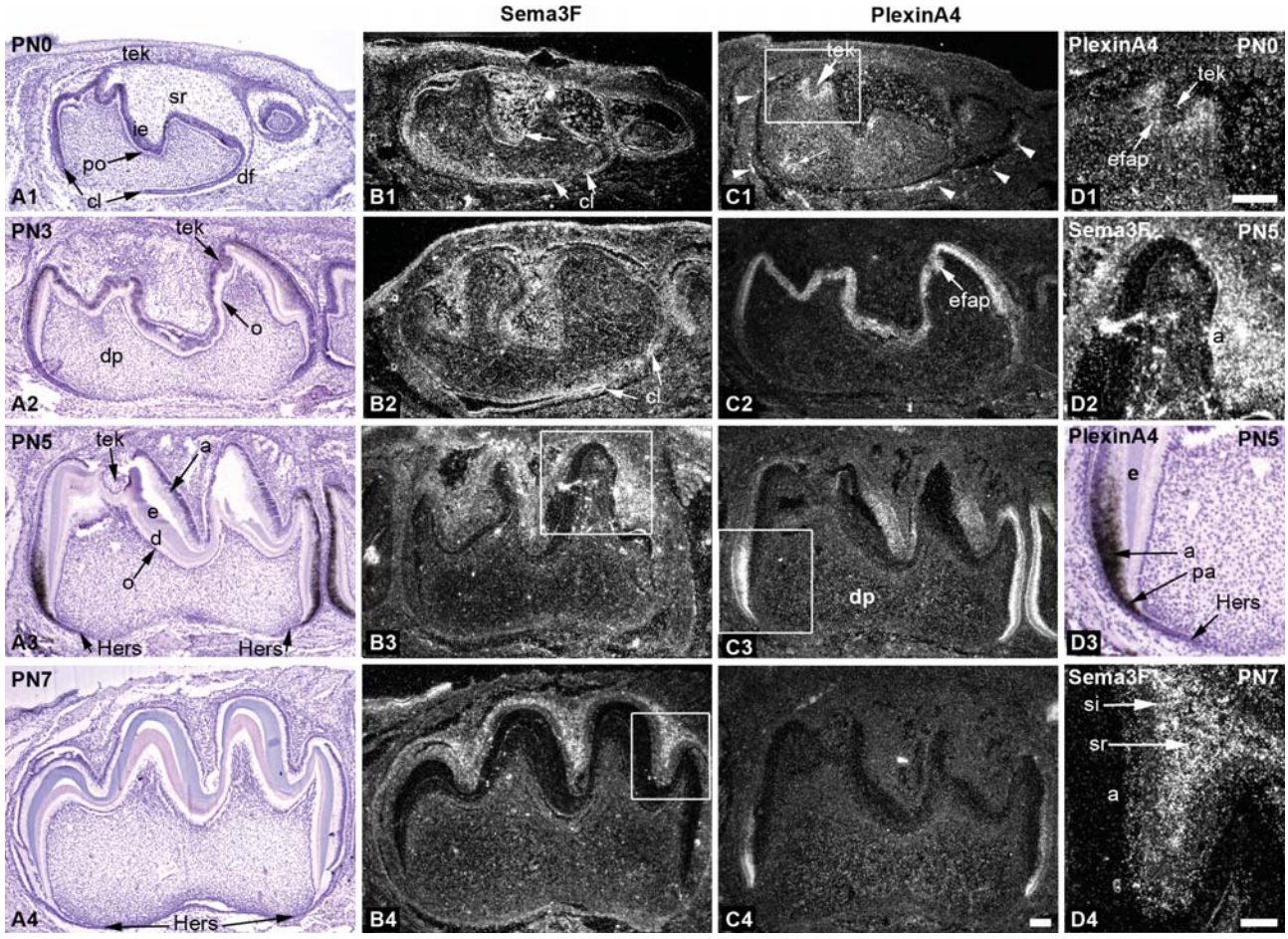

Figure 1. Expression of Sema3F and PlexinA4 mRNAs in postnatal day 0 (PN0), 3 (PN3), 5 (PN5) and 7 (PN7) mouse tooth germs. Bright (A1–A4 and D3) and dark-field (B1–C4, D1–D2 and D4) images of the sagittal sections of the first mandibular molars. (D1–D4 show higher magnifications of the areas marked with frames. Arrow in (B1) indicates *Sema3F* expression in the inner dental epithelium. In (C1) arrowheads indicate *PlexinA4* expression in the alveolar bone and a thin arrow indicates PlexinA4 expression in the dental papilla between the cervical loops. Bright-field images shown (A1–A4) are taken from the sections hybridized with PlexinA4 (C1–C3) and Sema3F (C4) probe. a, ameloblasts, cl, cervical loop; d, dentin; df, dental follicle; dp, dental pulp; e, enamel; efap, enamel-free area preameloblasts; ie, inner enamel epithelium; o, odontoblasts; pa, preameloblasts; po, preodontoblasts; si, stratum intermedium; sr, stellate reticulum; Hers, Hertwig's epithelial root sheath; tek, tertiary enamel knot. Scale bars: 100 μm in C4 applies to A1–C4; 50 μm in D4; 100 μm in D1 applies to D1 and D3.

signal in the dental papilla and in the enamel organ presumably representing blood vessels, were visible already at this stage (Figure 2B1). At PN3, *Npn1* expression pattern, similar to that observed at PN0 continued in the enamel organ and pulp (Figure 2B2). Additionally, some Npn1 mRNAs were now detected in the secreting ameloblasts (Figure 2B2). At PN5, the expression of Npn1 correlated to that of PN3. Transcripts were seen in the secretory ameloblasts, in the cuspal slopes, efa ameloblasts and in blood vessels (Figures 2B3 and B5). At PN7, *Npn1* expression was notably down-regulated from the dental pulp. *Npn1* mRNAs persisted in the pulpal blood vessels, but had disappeared in post-secretory ameloblasts (Figure 2B4). Besides the developing tooth proper, Npn1 transcripts were visible in the developing alveolar bone in all studied stages. Moreover, transcripts were seen around the inferior alveolar nerve at PN5 (not shown).

At PN0 Npn2 mRNAs were present in the epithelial enamel organ including the preameloblasts (Figures 2C1 and A5). A prominent expression was also apparent in the dental pulp, especially in pulp horns including the preodontoblasts and underlying subodontoblastic area (Figures 2C1 and A5). At PN3, the expression of Npn2 was largely down-regulated from the epithelial enamel organ and mesenchyme (Figure 2C2). However, a strong signal persisted in the preameloblasts and the efa ameloblasts next to the TEK (Figures 2C2 and C5). In addition, transcripts were seen in the dental pulp cells next to the cervical loops and in the site of the future pulp floor (Figure 2C2). Expression of *Npn2* continued the efa ameloblasts at PN5 and in the dental pulp cells next to the developing pulp floor (Figure 2C3). As in earlier stages the mesenchymal dental follicle and developing alveolar bone surrounding the tooth germ showed a prominent *Npn2* signal. A notable *Npn2* expression

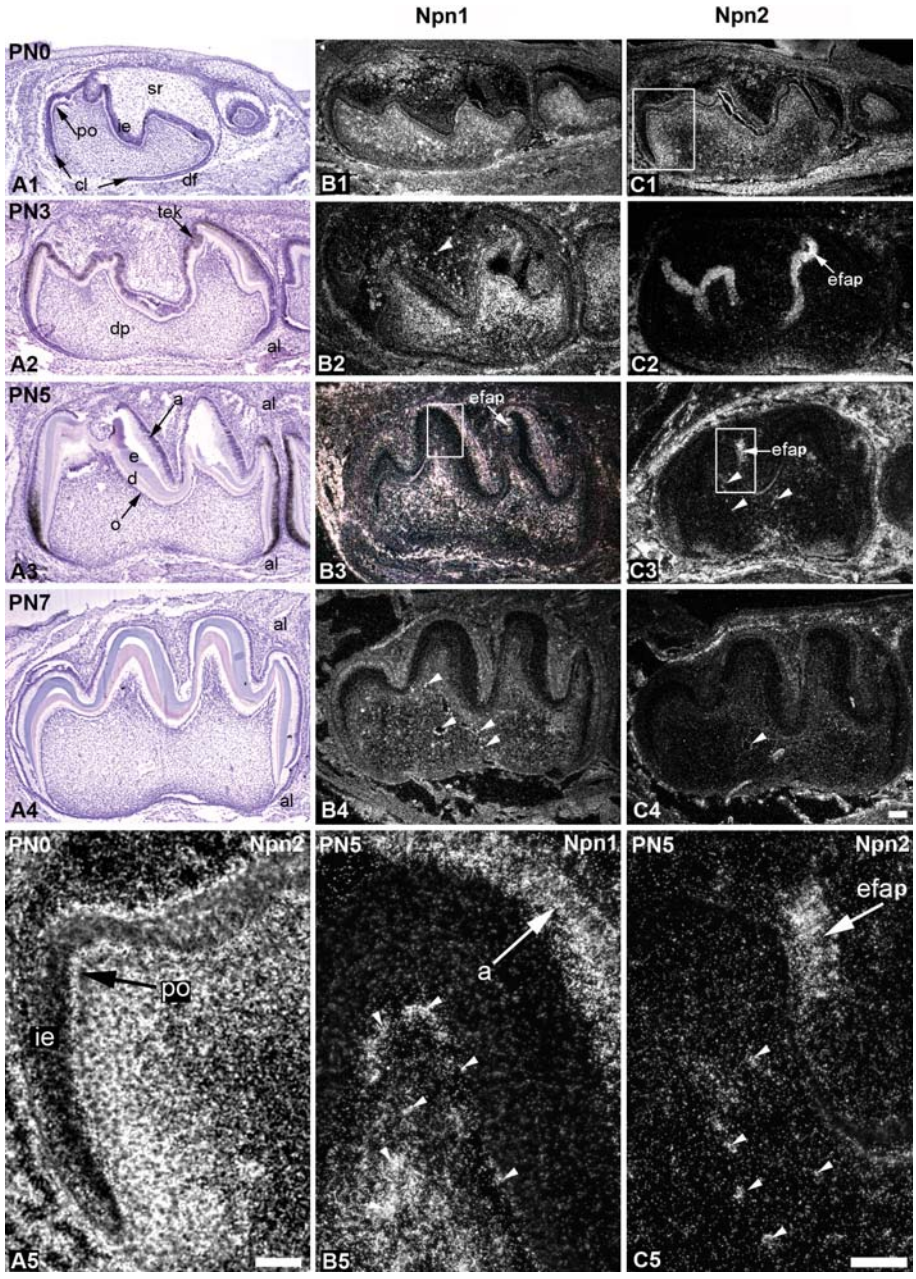

Figure 2. Expression of Npn1 and Npn2 mRNAs in postnatal day 0 (PN0), 3 (PN3), 5 (PN5) and 7 (PN7) mouse tooth germs. Bright (A1–A4) and dark-field (B1–C5) images of the sagittal sections of the first mandibular molars. (A5, B5 and C5) show higher magnifications of the areas marked with boxes. Arrowheads indicate gene expression in some selected blood vessels. Bright-field images shown (A1–A4) are taken from the sections hybridized with PlexinA4 probe (Figure 1C1–C3) and Sema3F (Figure 1C4). a, ameloblasts; al, alveolar bone; cl, cervical loop; d, dentin; de, dental epithelium; df, dental follicle; dp, dental pulp; e, enamel; efap, enamel-free area preameloblasts; ie, inner enamel epithelium; o, odontoblasts; po, preodontoblasts; sr, stellate reticulum. Hers; Hertwig's epithelial root sheath; Tek, tertiary enamel knot. Scale bars: 100 μm in C4 applies to A1–C4; 100 μm in A5; 50 μm in C5 applies to B5 and C5.

was also seen in small clusters of cells apparently representing blood vessels in the dental pulp as shown in higher magnification for PN5 tooth in Figure 2C5 (arrowheads). At PN7 Npn2 expression was restricted to the alveolar bone and blood vessels (Figure 2C4). As reported earlier the hair follicles were signal positive ([22], data not shown).

Expression of neuropilin and plexinA3 and -A4 receptors in the trigeminal ganglion

To assess whether the Sema3F, neuropilin, plexinA3 and -A4 might play a role in the establishment of tooth nerve supply, we investigated the expression of the receptor mRNAs in the trigeminal ganglion at the timepoint when the pioneer dental sensory

axons have entered into the dental pulp of the mouse mandibular first molar tooth [30,31]. *Npn1* showed prominent expression in trigeminal ganglion neurons whereas a weaker plexinA3 and -A4 signal was seen in a sub-set of neurons. No specific expression of *Npn2* was observed in the neurons (Figures 3A1–B7).

Discussion

The tooth is one of distinctive vertebrate organs that begins to develop with a simple epithelial bud, later undergoing a complex morphogenesis. To date, the mouse molar tooth germ has proven to be a useful model of studying molecular regulatory mechanisms incorporating axonal guidance and patterning with organ formation [2,3]. The class 3 semaphorins are important regulators of vertebrate axon guidance, cell migration and blood vessel development [5]. Recently, Sema3A and Npn1 were shown to control dental axon navigation, demonstrating an essential role for semaphorin signaling during tooth development [14]. To further address the potential roles of the semaphorin/neuropilin/plexin system during tooth organ formation and establishment of early tooth nerve supply, we investigated cellular mRNA expression patterns of Sema3F, Npn1, Npn2 and plexinA3 and -A4 receptors in the developing mouse mandibular first molar tooth germ from PN0 bell stage to a fully-formed crown at PN7. Moreover, we studied the expression patterns of the receptors in the PN5 trigeminal ganglion when the first dental trigeminal axons have entered the dental pulp [30,31]. We found that *Sema3F*, *Npn1*, *Npn2* and *plexinA4* exhibit developmentally regulated expression patterns in both epithelial and mesenchymal components of the tooth, whereas *plexinA3* was not observed. *Npn1* and *plexinA4* were also expressed in a sub-set of the trigeminal ganglion cells while *Npn2* and *plexinA3* was hardly discernible.

Sema3F may control tooth innervation

The tooth receives rich sensory as well as sympathetic innervation from trigeminal and superior cervical ganglion, respectively [32–34]. There are two major dental target areas of trigeminal nerve endings. One is the dental pulp, where nerve fibers form a dense network in the subodontoblastic region and innervate

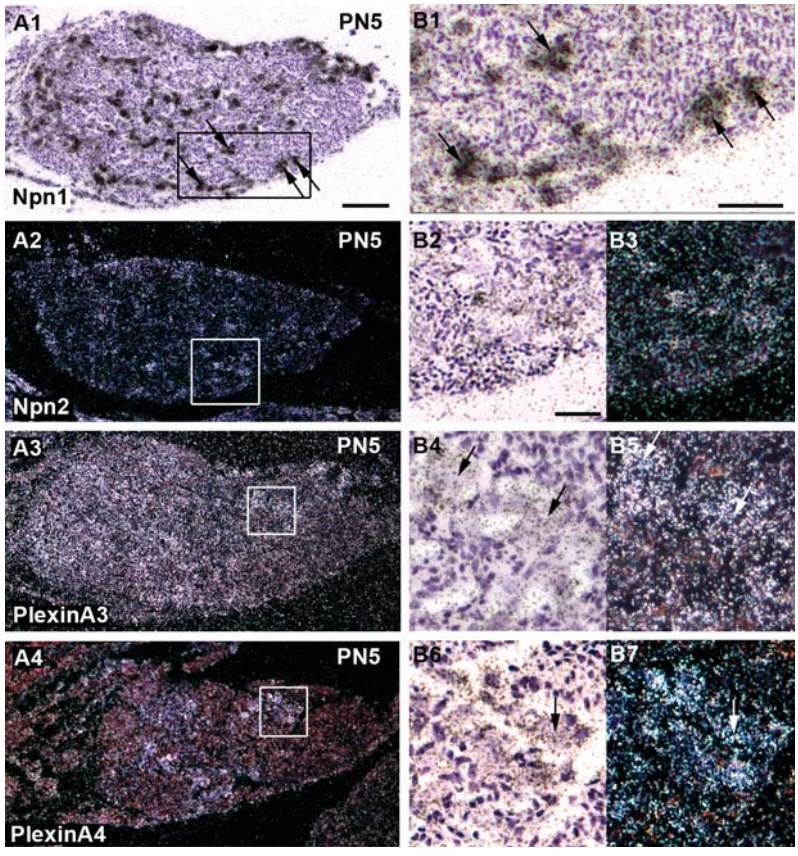

Figure 3. Expression of Npn1 (A1, B1), Npn2 (A2, B2, B3), PlexinA3 (A3, B4, B5) and PlexinA4 (A4, B6, B7) mRNAs in 5-day postnatal (PN5) trigeminal ganglia. Bright- (A1, B1, B2, B4, B6) and dark-field (A2-A4, B3, B5, B7) images. (B1–B7) show higher magnification of the areas marked with frames. Silver grain clusters (black arrows) in the (A1) and (A2) bright-field images show *Nnp1* expressing neurons. (A2–B5) PlexinA3 and plexinA4 transcripts are seen in a sub-set of trigeminal ganglion neurons while no specific Npn2 expression is seen in the cells (A4–B7). Arrows indicate some selected neurons showing positive hybridization signal. Scale bars: 100 μm in A1 applies to A1–A4; 50 μm in B1; 25 μm in B2 applies to B2–B7.

odontoblast and pre-dentin layer and the inner part of dentin [32]. Another is the dental follicle derived periodontal ligament, which connects the roots of the tooth to the alveolar bone and jaw [32]. In the mouse mandibular first molar, the dental nerve supply of the pulp starts by sensory innervation after deposition of enamel postnatally [30,31]. This is followed by ingrowth of sympathetic nerve fibers immediately after commencement of the root formation [30,31]. There is accumulating evidence that growing nerve fibers are directed to their specific targets by attractive and repulsive molecular cues located along their immediate surroundings [2]. Recently semaphorin signaling was shown to serve an essential role in this process, as Sema3A was demonstrated to repel dental sensory axons and regulate timing and patterning of the sensory innervation of the tooth [14]. In the present study, Sema3F transcripts were found in the cervical part of the dental pulp between cervical loops prior to nerve fiber ingrowth and later when the pioneer nerve fibers have already entered the pulp *Sema3F* expression was not visible any longer. This expression domain correlates to that of Sema3A repellant, which has been proposed to prevent premature ingrowth of the nerve fibers into the dental pulp. There is a substantial body of evidence that Sema3F acts principally through neuropilin-2 and plexinA3 receptors and confer repulsive responses as shown for growing sympathetic, motor, olfactory and hippocampal axons [19,21–23]. Besides these receptors, Sema3F also binds to Npn1 and plexinA4 receptors [26,35]. Although Sema3F did not repel embryonic sensory trigeminal and dorsal root ganglion axons in *in vitro* assays [19], mice deficient for Sema3F, Npn1, Npn2, plexinA3 and plexinA3/A4 appear all to show defects in peripheral trigeminal nerve projections [19,22,23,26,27,36]. Moreover, mice deficient to Npn1 showed defasciculation of dental trigeminal axons [14]. Besides being reported in embryonic stages [6,7,14,15,18,20,22,27,37–43], our novel data here shows that PlexinA4 and -A3 and Npn1 mRNAs are also expressed in the postnatal trigeminal ganglion during sensory trigeminal axon ingrowth into the dental pulp at PN5 [30,31]. However, because Npn2 was not observed in the neurons, it is therefore tempting to speculate that Sema3F, as proposed earlier for Sema3A, may contribute to regulation of trigeminal dental nerve fiber penetration into the dental pulp postnatally by acting as an axonal repellant possibly through Npn1/plexinA4 complex or plexinA4 alone [26]. It has also been suggested that besides plexins and neuropilins other transmembrane receptors may contribute to semaphorin responses [26]. Finally, because Sema3F has been shown to repel superior cervical ganglion axons it may also control sympathetic innervation of the tooth possibly by regulating ingrowth of the sympathetic nerve fibers into the pulp that follows sensory innervation at around PN9 in mice [31]. We also noted *Sema3F* in the stellate reticulum and stratum intermedium cells of the enamel organ at all stages studied. The enamel organ becomes vascularized before eruption of the tooth into the oral cavity commences, but in contrast to the dental pulp, it does not receive nerve fibers. Thus, it is tempting to speculate that Sema3F might selectively act as an axonal repellant to prevent nerve fiber entering the enamel organ.

Sema3F may control differentiation and function of the ameloblasts

In situ hybridization analysis revealed that *Sema3F*, *Npn1*, *-2* and *plexinA4* receptors were expressed in sites that did not correlate to the innervation of the tooth. At the bell stage, terminal differentiation of the enamel-forming ameloblasts takes place and the final crown shape is determined by the enamel as well as dentin formation. We observed *plexinA4*, *Npn1* and *Npn2* receptors in the preameloblasts and/or secreting ameloblasts, whereas no signal was seen in the postsecretory ameloblasts after the termination of enamel secretion. *Sema3F*, in turn, was observed in the inner enamel epithelium, stratum intermedium and stellate reticulum. Moreover, Sema3F and *plexinA4*, *Npn1* and *Npn2* were colocalized in the efa preameloblasts/ameloblasts, which fail to produce enamel at the tips of the cusps and the transverse connecting lophs of the molar tooth germ [29]. Hence, these results suggest that autocrine and possible paracrine Sema3F signaling through plexinA4 and Npn1 and/or Npn2 receptor complex may control the differentiation of the ameloblast-cell lineage and regulate the secretory functions and maintenance of functional properties of the ameloblasts. The expression of *Npn1* and *Npn2* in the preodontoblasts suggests that they may possibly serve roles in odontoblast determination and differentiation as well.

Sema3F and neuropilins in non-neuronal tooth supporting tissues

There is an increasing body of evidence that semaphorin signaling controls blood vessel and bone formation. Indeed, *Npn1* and *Npn2* mRNAs were found in the blood vessels of the dental pulp and epithelial stellate reticulum. Neuropilin-1 and -2 are also receptors for vascular endothelial growth factor (Vegf) [44,45]. Sema3F has been earlier shown to be expressed in the endothelial cells of the blood vessels and shown to repel endothelial cells *in vitro* [24]. Similarly, Npn1 has been reported in the endothelial cells of arteries [44,46] and Vegf-Npn1 signaling in endothelial cells is required for vasculogenesis [47,48]. Both Sema-Npn-1 signaling and Vegf-Npn1 signaling are also critical for heart development [44,47,48]. In contrast, Npn2 expression in the vascular system is confined to veins and lymphatic vessels and mice deficient for Npn2 suggest that it is required for the formation of small lymphatic vessels and capillaries

[49]. Thus, the expression of *Npn1* and *Npn2* suggests that they are involved in the development of the vascular system in the tooth, but that they may serve different roles. Whereas Npn1 may be involved in formation of arterioles Npn2 is suggested to regulate development of venules, small lymphatic vessels and capillaries of the tooth.

We additionally observed *plexinA4*, *Npn1* and *Npn2* in the developing bone surrounding the developing tooth germ. Earlier Npn1 transcripts have reported to be expressed in osteoblasts but down-regulated as osteoblasts differentiate into osteocytes [50]. Furthermore, *Npn2* is expressed in the developing bone during embryogenesis [18]. Npn1 mediated Sema3A signaling appears to control osteogenesis as shown by bone defects in Sema3A null mutant mice [37] and extra digits in Npn1 over-expression mice [46]. Our results suggest that Npn1, -2 and PlexinA4 mediated signaling may regulate the alveolar bone formation. However, because no specific Sema3F hybridization signal was observed in the developing alveolar bone, other molecules are proposed to act as functional ligands for the receptors during osteogenesis. One putative candidate is Sema3A, which is prominently expressed in the mandibular and alveolar bone from the earliest stages of their development [12,14].

In summary, our data show that the *Sema3F* as well *Npn1*, *Npn2* and *plexinA4* receptors exhibit developmentally regulated expression patterns during tooth formation whereas plexinA3 transcripts were not observed. The cellular expression of Sema3F suggests that it may act as a target-derived axonal chemorepellant controlling tooth innervation. In addition, the expression of *Sema3F* and the receptors *Npn1*, *-2* and *plexin-A4* in sites unrelated to axon growth, in particular in the ameloblast cell lineage, blood vessels and alveolar bone suggests that they may also serve non-neuronal roles in odontogenesis. Further analyses such as investigation of transgenic mouse models will elucidate their *in vivo* function in formation of tooth proper and it's supporting tissues.

Materials and methods

Animals and preparation of tissues

Animal use was approved by the Department of Biomedicine in the Medical and Dental Faculty at the University of Bergen under the surveillance of the Norwegian Animal Research Authority. Albino mice (BomTac: NMRI) were mated overnight and the appearance of vaginal plug was taken as day 0 of embryogenesis. The day the litter was born was taken as postnatal day 0 (PN 0). Mice in postnatal day 0 (PN0), 3 (PN3), 5 (PN5) and 7 (PN7) were sacrificed by decapitation. Mandibles and heads were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C after 1–2 days. Altogether five teeth were used in each stage for experiments. Mandibles and heads were demineralized in 4% PFA/12.5% EDTA (ethylenediaminetetraacetic acid) at room temperature for 1–6 weeks depending on the postnatal stage. Thereafter, tissues were rinsed in running tap water for 2–3 days, dehydrated and embedded in paraffin. Sagittal sections of 7 μm were cut, placed on slides, dried overnight at 37°C and stored at 4°C.

In situ hybridization

Radioactive *in situ* hybridization was performed as described previously [51]; 2.9 kb mouse Sema3F, 400 bp plexinA3, 719 pb plexinA4 as well as 1.1 kb Npn1 and 1.1 kb Npn2 plasmids, which have been described earlier [12,15,20], were used for *in vitro* transcription of 35S-UTP-labeled antisense and sense probes. Sections were exposed for 3 weeks. No specific hybridization signals were detectable in tissue sections hybridized with control sense probes (data not shown).

Images

All sections were examined and photographed in dark-field and bright-field using a Zeiss Axioskop 2 (Carl Zeiss Jena GmBH, Jena, Germany) microscope. Objectives with magnification ×5 and ×10 were used. The images were captured using a Spot Insight digital camera (Diagnostic Instruments Inc. Sterling Heights, MI). Processing of the images and image plates were performed using Adobe Photoshop 6.0.1 software (Adobe Systems, San Jose, CA).

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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