Semaphorin 3A and Class 4 Semaphorins in Tooth Innervation and Development

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"*This work is dedicated to my family* "

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ABBREVIATIONS

ABC	Avidin biotin complex
AEC	3-amino-9-ethyl carbazol
BABB	Benzyl alcohol/benzyl benzoate
Bdnf	Brain derived neurotrophic factor
Bmp	Bone morphogenic protein
Barx	BarH-like homeobox
CT	Computed tomography
CEJ	Cementoenamel junction
CNS	Central nervous system
CRMP	collapsin response mediator protein
DAB	3,3´ diamino benzadine
Dlx	Drosophila distal-less gene
DDT	Dithiothreitol
DAB	3,3´ diaminobenzidine
E	Embryonic day
EDA	Ectodysplasin
EDTA	Ethylenediaminetetraacetic acid
Ek	Enamel knot
ELISA	enzyme-linked immune sorbent assay
Fgf	Fibroblast growth factor
Gdnf	Glial cell line-derived neurotrophic factor
HERS	Hertwig's epithelial root sheath
HRP	Horse radish peroxidase enzyme
IHC	Immunohistochemistry
ISH	*In situ* hybridisation
K14	Keratin 14
kDa	Kilodalton
Lef	Lymphoid enhancer factor
mRNA	Messenger ribonucleic acid

Msx	Vertrbrate homologue of *Drosophila* muscle segment homeobox
Ncam	Neural cell adhesion molecule
NF-200	Neurofilament-200
Ngf	Nerve growth factor
NMRI	Naval Medical Research Institute (USA)
Npn	Neuropilin
Nt	Neurotrophin
OCT	Optimal cutting temperature
Pax	Paired box –containing transcription factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pek	Primary enamel knot
PFA	Paraformaldehyde
PGP9.5	Protein gene product 9.5
PN	Postnatal day
PNS	Peripheral nervous system
Sek	Secondary enamel knot
Sema	Semaphorin
Sema3s	Class 3 semaphorins
Sema4s	Class 4 semaphorins
Shh	Sonic hedgehog
SP	Substance P
Tek	Tertiary enamel knot
Tgfß1	Transforming growth factor beta-1
Tim-2	T cell immunoglobulin and mucin-domain-containing 2
Trk	Tyrosine kinases
WB	Western blot
Wnt	vertebrate homologue of *Drosophila* Wingless

SCIENTIFIC ENVIRONMENT

The research work of this thesis was performed at the Craniofacial Developmental Biology Group, Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen, Norway, under the supervision of Professor Päivi Kettunen as the main supervisor, and Professor Keijo Luukko as the co-supervisor. The project was supported by The Faculty of Medicine and Dentistry, University of Bergen.

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SUMMARY

Background: Dental trigeminal axon elongation, navigation and patterning occur in a controlled manner that is intimately linked to tooth shape formation and cell differentiation. Development of tooth results from sequential and reciprocal molecular interactions between epithelial and mesenchymal tissues. Semaphorin family of secreted and membrane-bound axonal growth cone guiding molecules regulates the development of the nervous system and also serves important non-neuronal functions. Many Semaphorins are expressed in the developing tooth germ and there is evidence that Semaphorin signalling regulates tooth innervation. **Objective:** To investigate mRNA expression of class 4 semaphorins and their PlexinB receptors in the developing mouse mandibular first molar, and to study further functions of Sema3A during odontogenesis. **Materials and methods:** Transgenic Sema3A-deficient mice in C57BL/6 and CD1 background as well as NMRI mice were used. *In situ hybridization* and immunohistochemistry was employed to localize mRNAs and neurites on tissue sections of embryonic and postnatal teeth. In addition, western blot was used to investigate presence of class 4 Semaphorins in postnatal mandibular first molar tooth germ and trigeminal ganglion**.** Computed tomography was applied to study adult teeth**.** **Results:** Sema4A and Sema4D as well as PlexinB1 and -B2 receptor mRNAs were expressed in the postnatal molar tooth germ. Sema4D, PlxnB1 and PlxnB2 proteins were also found in the postnatal molar tooth germ and trigeminal ganglion. *Sema3A* showed dynamic expression in the developing mandibular incisor. Analysis of the Sema3A-deficient mice revealed that Sema3A signaling is required for proper innervation of the embryonic and postnatal incisor tooth germ as well as postnatal molar whereas no apparent histomorphological defects in the development of tooth germs were observed. **Conclusions:** The expression domains of the class 4 semaphorins suggest that they may serve both neuronal and non-neuronal functions during odontogenesis. Sema3A controls innervation of the pulp and periodontium during tooth development. The putative, neuronal and non-neuronal roles of the semaphorins, which may be redundant during odontogenesis, remain to be analysed in the future.

LIST OF ARTICLES

The present thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

Article I

Kyaw Moe**, Anjana Shrestha**, Inger Hals Kvinnsland, Keijo Luukko and Päivi Kettunen (2011). Developmentally regulated expression of Sema3A chemorepellent in the developing mouse incisor. Acta Odontologica Scandinavica.70, 184-189.

Article II

Kyaw Moe*, Angelina Sijaona*, **Anjana Shrestha***, Päivi Kettunen, Masahiko Taniguchi and Keijo Luukko (2012). Semaphorin3A controls timing and patterning of the dental pulp innervation. Differentiation. 84, 371-379.

Article III

Anjana Shrestha*, Kyaw Moe*, Keijo Luukko, Masahiko Taniguchi and Päivi Kettunen. (2014) Sema3A chemorepellent regulates the timing and patterning of the dental nerves during the development of incisor tooth germ. Cell and Tissue Research. 22 (published online April, 22nd 2014).

Article IV

Anjana Shrestha, Keijo Luukko and Päivi Kettunen. Dynamic expression of Class 4 semaphorins and PlexinB receptor mRNAs in the early postnatal mouse molar suggests neuronal and non-neuronal functions during odontogenesis. (Manuscript to be submitted).

*Equal contribution

1. INTRODUCTION

1.1 Vertebrate dentition

Teeth are one of the characteristics of very many extant vertebrates. Fish, amphibians and reptiles exhibit continuous renewal and replacement of their teeth (polyhyodont dentition) (Whitlock and Richman, 2013). Mammals exhibit two generations of teeth (diphyodont dentition) and rodents one (monophyodont dentition). Birds have no teeth at all (anodont), although reactivation of the early odontogenic pathway is possible in the chicken oral epithelium with an external inducer (Sire et al., 2008). Animals use teeth for feeding, grooming and defending (Szalay and Seligsohn, 1977). In humans, dentition is not merely needed for mastication, but also for proper articulation. Intact, well-aligned teeth in man are additionally considered pleasing and therefore serve an aesthetic function. The type of dentition reflects animal diet (Jernvall and Thesleff, 2012) (Rodrigues et al., 2013). Man is omnivorous and has four various types of teeth: incisors, canines, premolars and molars.

1.2 Human tooth

Anatomically a tooth consists of a crown and a root (Fig. 1). A crown denotes the visible part in the oral cavity. The anatomic crown of a tooth is covered by enamel above the cementoenamel junction (CEJ). The majority of the crown is composed of dentin with the pulp chamber inside. The anatomical root is located below the CEJ and is covered with cementum (Nanci and Ten Cate, 2013), which is connected with periodontal fibers to the alveolar bone. A human tooth may have one, two or three roots. Like with the crown, dentin composes most of the root, which usually has 1-2 root canals. An apical foramen is an opening in the apex of root and functions as a major passage for blood vessels and nerves into the pulp (Avery, 1994). Each incisor has an incisal edge, which is used for cutting food. The canine has a single cusp used for grasping and tearing food.

Posterior teeth, premolars and molars, have 2-3 and 4-5 cusps, respectively, and are used for crushing and grinding food. Development of the dentition takes several years in man. The dental lamina for the deciduous dentition appears during weeks 7-8 of the embryonic stage and wisdom teeth in permanent dentition erupt at the age of about 20 years (Nelson et al., 2010).

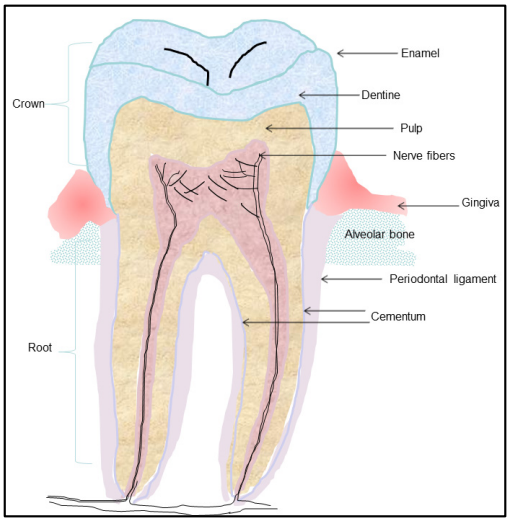

Figure 1. Human molar tooth. Modified from (http://e.wikipedia.org/wiki/Human_tooth).

1.3 Tooth development

1.3.1 Anatomical development of mouse mandibular first molar

Most of our knowledge concerning the molecular regulation and genetics of mammalian odontogenesis has been obtained from investigation on mouse dentition (Klein et al., 2013). In particular, the mouse mandibular first molar has been an excellent model system to reveal general molecular mechanisms regulating formation of organs and their supporting tissues (Thesleff et al., 1995b) (Jernvall and Thesleff, 2000). Each half of a

mouse jaw has a single incisor and three molars. Between an incisor and molar teeth there persists a region devoid of teeth called a diastema. This region contains rudimentary tooth germs arrested at the bud stage and eventually degenerated by apoptosis in the maxillary process (Keranen et al., 1999) (Klein et al., 2013). The histomorphological formation of a tooth is traditionally divided into three overlapping phases namely initiation, morphogenesis and cell differentiation (Kollar and Lumsden, 1979). The first evidence of molar tooth development in mice is seen at around embryonic (E) day 11 when a local thickening of oral epithelium is detected (Fig. 2). Dental epithelial cells further proliferate into an individual globular swelling, bud, which grows into the jaw ectomesenchyme, the origin of which is from the neural crest that condenses around the bud (E12-13, bud stage). At E14-15, the cervical loop buds off from the dental epithelium and subsequently the dental epithelium acquires the cap shape (cap stage). From the cap stage onwards the dental epithelium is defined as an enamel organ, which consists of the outer dental epithelium, stellate reticulum cells, and inner dental epithelium (Lesot and Brook, 2009). Dental papilla is seen adjacent to the inner dental epithelium of the enamel organ and both of them are surrounded by mesenchymal dental follicle cells. During the following bell stage (E16 onwards) tooth specific occlusal topography start to emerge when folding of the inner dental epithelium increases, and consequently the tooth germ undergoes further morphodifferentiation and histodifferentiation forming distinct tooth specific cell types (Hay, 1961). The developing dental epithelium adjacent to the dental mesenchyme houses clusters of undifferentiated cells defined as enamel knots, which are suggested to be involved in establishment of tooth shape. Primary enamel knots are present in the bud and cap stage, secondary enamel knots appear in the bell stage and tertiary enamel knots are seen in a later bell stage (Jernvall et al., 1994) (Luukko et al., 2003).

After the general crown morphology has been established and the last ameloblasts have started to produce enamel, the cervical loop does not exist any more. Stellate reticulum and stratum intermedium cells disappear and the remaining two cell layers of inner and outer dental epithelium form Hertwig's epithelial root sheath (HERS). HERS induces differentiation of odontoblasts in the root and thus contributes to the formation of the

root (Tummers et al., 2007). Once HERS has induced odontoblast differentiation, it is transformed into Malassez' epithelium, which degenerates to become Malassez' epithelial rests. Neural crest derived undifferentiated dental follicle cells next to the root dentin differentiate into cementoblasts, which secrete the root dentin covering cementum (Tummers et al., 2007). Furthermore, fibroblasts in the dental follicle produce principal collagen fibers, which anchor the root to the alveolar bone. When two-thirds of the root has been formed the tooth starts to erupt into the oral cavity and subsequently the root formation is completed (Kettunen et al., 2005) (Miletich and Sharpe, 2003). Tooth formation is accompanied by development of blood and nerve supply as well as intramembranous ossification of alveolar bones.

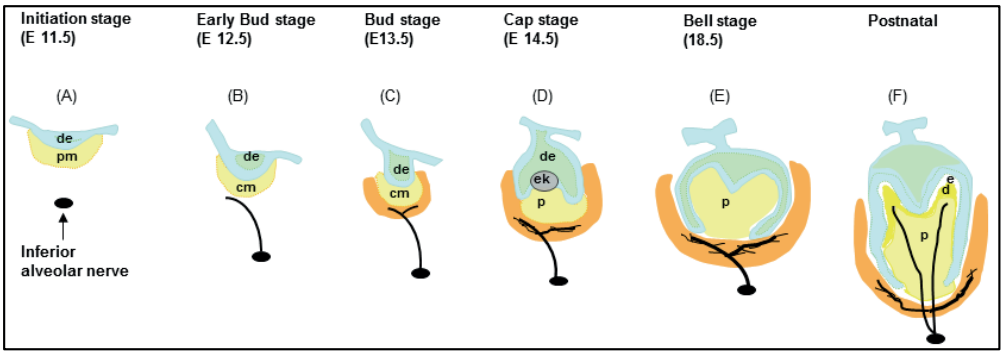

Figure 2. Schematic illustration of the development of mouse molar tooth germ and its innervation. Abbreviations: de, dental epithelium; pm, presumptive dental mesenchyme; cm, condensed dental mesenchyme; ek, enamel knot; p, pulp; e, enamel; d, dentine. Nerve fibers are indicated in black. Modified from (Luukko et al., 2005a).

1.3.2 Anatomical development of mouse mandibular incisor

In contrast to the human incisor, the mouse incisor erupts throughout the life due to activity of stem cells in the cervical loop (Harada et al., 1999) (Schonfeld and Slavkin, 1977). Thus, the mouse incisor is a useful model organ in which to study stem cells in organogenesis. Histologically, the development of the mouse incisor begins at E12 as a

thickening of the dental epithelium, which further invaginates into the jaw mesenchyme and forms a tooth bud by E13. By E14 the tooth germ consists of a cap-shaped enamel organ and mesenchymal dental papilla surrounded by the mesenchymal dental follicle cells. The cervical loop in the labial side of the enamel organ grows more extensively than in the lingual side already at the cap stage and continues to do so at the bell stage in a proximal direction. Consequently, the whole tooth germ rotates antero-posteriorly and runs parallel to the long axis of the mandible (Fig. 10) (Wang et al., 2004) (Hay, 1961). The incisor is asymmetric (along the long axis) due to different histomorphology of the labial and lingual sides. Dentin is formed in the lingual side of the incisor that resembles the root of the molar tooth (root analogue), whereas in the labial side the dentin is covered by enamel (crown analogue) (Amar et al., 1986). Due to asymmetric enamel distribution, incisors are prone to continuous wear along the incisal edge and a life-long regeneration is required for the maintenance of an incisor tooth in the cervical loops (Jussila and Thesleff, 2012).

1.3.3 Odontoblast and ameloblast differentiation

Tooth specific cell differentiation is governed by the sequential and reciprocal interactions between epithelial and mesenchymal tissues (Lesot et al., 2001) (Thesleff et al., 1995a). The tissue interactions have been shown to be mediated in particular by growth factors and their signalling receptors. At the bell stage, the inner enamel epithelium cells start to differentiate into preameloblasts, and this induces differentiation of preodontoblasts that further differentiate into odontoblasts (Lesot et al., 2001) (Ruch et al., 1995) (Thesleff et al., 2001). These form a continuous cell layer at the junction between predentin and pulp (Ruch et al., 1995). As odontoblast differentiation commences in the area of the presumptive tooth cusps, underneath the secondary enamel knots, it has been suggested that signals from the secondary knots may regulate this process (Thesleff et al., 2001). Odontoblasts secrete predentin, which is biomineralized to form dentin. The growth factors stimulating the odontoblast differentiation *in vitro* include transforming growth factor (Tgf) beta superfamily

signals, fibroblast growth factors (Fgf) and insulin–like growth factors (Igf1) together with heparin or fibronectin (Ruch et al., 1995) (Martin et al., 1998) (Unda et al., 2000). Shh (sonic hedgehog) contributes to organization of the odontoblast layer (Dassule et al., 2000). In addition, Tgfß1 combined with either Fgf1 or Fgf2 stimulates polarization of odontoblasts, and Tgfß1 and Fgf1 together induce secretion of predentin-like collagen matrix *in vitro* (Unda et al., 2000). Bmp2, -4 and -7 have been reported to induce osteodentin or tertiary dentin formation *in vivo* (Iohara et al., 2004) (Six et al., 2002) (Rutherford et al., 1994) (Nakashima, 1994). In addition, there are autosomal dominant genetic conditions caused by mutations in type I collagen and dentin sialophosphoprotein genes, which give rise to various forms of abnormal dentine structure (Barron et al., 2008).

Final differentiation of ameloblasts and secretion of enamel occurs after initiation of mineralized dentin production (Thesleff and Hurmerinta, 1981) (Boukari and Ruch, 1981). It is preceded by breakage of the basement membrane, which allows interactions between preameloblasts and predentin to occur (Thesleff and Hurmerinta, 1981) (Ruch et al., 1995) *In vitro* and *in vivo* studies have provide evidence that odontoblast-secreted Tgfß1, Bmp2 and Bmp4 stimulate ameloblast differentiation (Coin et al., 1999) (Wang et al., 2004). In addition to signaling from the dental pulp cells, signaling within the inner dental epithelium mediated by Shh is needed for the proper cytodifferentiation of ameloblasts *in vivo* (Dassule et al., 2000) (Gritli-Linde et al., 2002). An abnormal formation of enamel has been observed on targeted genetic inactivation of Fgfr1 in ameloblasts (Takamori et al., 2008). In addition, micro-RNAs regulate ameloblast differentiation and enamel secretion (Michon et al., 2010). Follistatin regulates ameloblast differentiation as shown in follistatin knockout mice in which abnormal enamel secretion occurs on both sides of the mouse incisor (Wang et al., 2004). In contrast, in follistatin overexpression mice no enamel is deposited (Wang et al., 2004). The final shape of the molar crown is determined when enamel secretion stops. In mouse molars proper enamel secretion does not occur in the cusp tips and, therefore, in mouse molars these are enamel-free. Ridges between the buccal and lingual cusps are also enamel-free (Luukko et al., 2003). Amelogenesis imperfecta a well-known example

of a human genetic disorder with a wide range of clinical phenotypes in enamel, caused by mutations in half a dozen genes (Mitsiadis and Luder, 2011). This condition affects the qualitative and quantitative properties of enamel.

1.3.4 Dental stem cells

Stem cells are generally defined as cells having the ability to perform self-renewal and multi-lineage differentiation (Fuchs and Chen, 2013). Cervical loop of the ever-growing mouse incisor has been shown to contain stem cells (stem cell niche) (Harada et al., 1999). Several signalling molecules and growth factors regulate the proliferation and differentiation of stem cells, and influence the incisors' stem cell niche in the cervical loop (Harada et al., 1999) (Thesleff et al., 2007). Mesenchymal Fgfs in particular Fgf10 regulate proliferation of the cervical loops (Harada et al., 1999) (Juuri et al., 2012). Fgf10 and Fgf3 controls proliferation of daughter cells, which become transient-amplifying cells in the inner dental epithelium in mouse incisor (Harada et al., 1999). In addition, Fgf signaling also regulate transition of crown to root in developing molar (Yokohama-Tamaki et al., 2006). A recent finding showed that Sox2 expressing epithelial cells play a major role in the renewal of tooth epithelial cell lineages (Juuri et al., 2012) and Fgf8 and certain miRNAs control stem cells (Juuri et al., 2012).

Later, various stem cell populations have also been reported in the adult tooth such as cells from the pulp of exfoliated deciduous and extracted adult teeth, dental follicle, periodontal ligament, and apical papilla (Abe et al., 2008) (Gronthos et al., 2000) (Miura et al., 2003) (Handa et al., 2002a) (Handa et al., 2002b) (Nosrat et al., 2014). Epithelial rests of Malassez give rise to enamel like tissues once cultured together with dental pulp *in vitro* (Shinmura et al., 2008). Stem cells from the apical part of the radicular pulp have the capacity to differentiate into odontoblasts and adipocytes (Sonoyama et al., 2006). Stem cells play a significant role in the regeneration of dentine and periodontal tissues (Yen and Sharpe, 2008) (Shi et al., 2005) (Miura et al., 2003) (Bluteau et al., 2008). Recently, the neurovascular bundle was identified as a

mesenchymal stem cell niche in adult incisor (Zhao et al., 2014). It is possible, that dental stem cells might be used for regeneration of dental tissues and whole teeth (Mitsiadis et al., 2012) (Jussila and Thesleff, 2012) (Steindorff et al., 2014) (Nosrat et al., 2014).

1.3.5 Molecular regulation of tooth formation

Organogenesis is characterized by coordinated proliferation, determination differentiation of distinct cell types, which contribute to the formation of specific functional three-dimensional structures, organs. Dental developmental biology research in particular using mouse tooth germs has established that inductive tissue interactions between oral ectoderm and neural crest derived mesenchymal cells regulate tooth formation. These interactions control different phases of tooth development including initiation, morphogenesis, and cell differentiation (Cobourne and Sharpe, 2003) (Jernvall and Thesleff, 2000) (Thesleff, 2003) (Tucker and Sharpe, 2004). *In vitro* culture studies with separated dental tissues, which are recombined, have provided evidence that the presumptive dental epithelium controls early odontogenesis after which the potential governing tooth formation becomes transferred into the dental mesenchyme (Mina and Kollar, 1987) (Lumsden, 1988) (Kollar and Baird, 1970). The type of the tooth has been proposed to be determined by the distinct expression domains of certain homeobox genes in the early first brachial arch mesenchyme. Their expression ("the odontogenic homeobox gene code") is suggested to provide a spatial code comparable to the Hox gene code along the anterior posterior body axis (Sharpe, 1995) (Tucker et al., 1998; Tucker and Sharpe, 1999) (Thomas et al., 1997).

The final shape of the multicuspid molar tooth crown is dependent on and controlled by the three epithelial signaling centres, the enamel knots (ek) the primary (Pek), secondary (Sek), and tertiary (Tek) enamel knots (Jernvall et al., 1994) (Thesleff and Jernvall, 1997) (Luukko et al., 2003). These successive clusters of non-proliferative cells appear in the dental epithelium. The Pek appears at the tip of the epithelial bud and

is transferred into the middle of the inner dental epithelium at the cap stage, which determines the location of the first cusp in the future tooth. Following enamel knots are defined as Seks, which appear at the sites of other cusps (Thesleff and Jernvall, 1997) (Jernvall et al., 1994). The Teks, which develop from the upper compartment of the Seks, become located next to the enamel free areas at the cusp tips in mouse (Luukko et al., 2003). These signaling centres express a number of signaling molecules associated with regulation of the tooth growth and the formation of final crown shape (Luukko et al., 2003) (Jernvall and Thesleff, 2000) (Thesleff and Jernvall, 1997). In addition, the Seks and Teks, and amebloblast-free ridge (AFR) signaling area between the lingual and buccal cusps are proposed to create integrated signaling areas. They are proposed to control the distance between the cusps as well as height of the cusp as well as define the enamel free areas of the multicuspid crown (Luukko et al., 2003).

Soluble signaling molecules have been shown to mediate tissue interactions, which control multiple cellular processes leading to the progression of the tooth formation (Thesleff and Jernvall, 1997) (Cobourne and Sharpe, 2010) (Klein et al., 2013) Majority of the key signaling molecules belong to conserved growth factor families such as Fibroblast growth factor (Fgf), Transforming growth factor (Tgf), Hedgehog (Hh) and Wnt families (Vainio et al., 1993) (Kettunen and Thesleff, 1998) (Thesleff and Jernvall, 1997) (Cobourne and Sharpe, 2010) (Klein et al., 2013). Secreted signaling molecules bind to their cognate cell surface receptors on the target cells and activate different intracellular signaling pathways and subsequently regulate transcription of target genes in the nucleus. Various signaling pathways are integrated into complex networks at different levels and may have synergistic as well as counteractive effects (Tummers and Thesleff, 2009). It is likely that the diversity of different tooth types and dental patterns is a result of meticulous and intricate adjustment of the conserved signaling pathways and networks during evolution (Tummers and Thesleff, 2009) (Jernvall and Thesleff, 2012).

Many of the key signaling molecules regulating odontogenesis show distinct, restricted expression in the enamel knot signaling centres. For instance enamel knot produced

Fgf4 signaling via Lef-1 transcription factor is crucial for tooth morphogenesis (Jernvall et al., 1994; Kratochwil et al., 2002). Enamel knot expressed signaling molecules has been shown to induce expression of different key genes in the dental mesenchyme including Msx1 and Pax9 (Thesleff and Nieminen, 1996) (Tucker and Sharpe, 2004), mutations of which is known to underlie dental agenesis in man (Vastardis et al., 1996) (Stockton et al., 2000). Mutations in a suppressor of canonical Wnt pathway AXIN2 and ectodermal dysplasia genes EDA, EDAR and EDARADD have been identified in familial oligodontia (Lammi et al., 2004) (Nieminen, 2009). Mutations in WNT10a give rise to isolated hypodontia, in which phenotype may vary from mild to severe (van den Boogaard et al., 2012). Recently, Pek and dental mesenchyme expressed Fgf3 influence on number of cusps in mouse and man (Charles et al., 2009). Moreover, mutations in several other genes have been reported in various syndromes where absence of teeth is a common feature (Nieminen, 2009) (Klein et al., 2013). Tooth malformations are characteristic also for many cilopathies, in which primary cilium-dependent integration of Hedgehog and Wnt signaling is disturbed (Liu et al., 2014). Conserved signaling families, which regulate tooth formation such as Wnt and Bmp appear to be reused in tooth replacement (Jernvall and Thesleff, 2012). Of note, trigeminal neurites are essential for tooth germ development in fish (Tuisku and Hildebrand, 1994). The possible role of innervation for the induction of tooth formation in mammals remains to be elucidated (Fried et al., 2000) (Luukko and Kettunen, 2014).

1.4 Nerve supply in mature tooth

The tooth is supplied by sensory and sympathetic nerve fibers originating from trigeminal and superior cervical ganglion, respectively (Hildebrand et al., 1995; Byers and Narhi, 1999). The existence of parasympathetic innervation in the tooth appears to be controversial (Olgart, 1996). The trigeminal ganglion has two peripheral branches, sensory ophthalmico-maxillary and mandibular branch, which consists of both sensory and motor fibers (Hildebrand et al., 1995). In mouse, the first mandibular molar, and the mesial root of the second molar are innervated by a branch from the inferior alveolar

nerve (IAN). A branch from the lingual nerve provides nerve supply to the distal root of the second molar and the third (Naftel et al., 1999). The IAN also gives off an incisor branch, which ramifies at the level of the second molar and forms a nerve plexus in the periodontal space around the incisor (Naftel et al., 1999). Branches from the plexus penetrate into the incisor pulp through apical opening (Naftel et al., 1999).

Trigeminal nerves have two main target areas in the tooth, the dental pulp and periodontal ligament (Fig. 3). The nerve bundles in the molar radicular pulp accompany large blood vessels (Hildebrand et al., 1995). In the coronal pulp close to the odontoblast layer sensory defasciculated neurites form subodontoblastic nerve plexus. Some of neurites become located within the odontoblast layer and generate marginal plexus between odontoblasts and predentin. In addition, some free nerve endings extend into the dentin tubuli up to 100-200 μm (Byers and Narhi, 1999). The number of sensory neuritis is highest in the pulp horn tips, and it diminishes gradually along the slopes of the cusps towards to crown-root junction. There is no nerve plexus in the floor of the crown pulp. In the radicular pulp, only a very few nerve fibers are seen in the odontoblast layer and dentin tubuli (Byers and Narhi, 1999) (Hildebrand et al., 1995). In the incisor, a few thin neurites has been reported to extend to the odontoblast layer (Naftel et al., 1999).

Dental pulp is mostly innervated by sensory, nociceptive myelinated A-beta, A-delta and unmyelinated C-fibers (Byers and Narhi, 1999) (Byers et al., 2003). A-delta fibers mediate acute, sharp pain and some of them pre-pain. A-beta fibers mediate pre-pain whereas C-fibers are induced by stimuli such as heating or cooling that get into the deeper pulp and produce dull pain (Byers et al., 2003). In addition to mediating painful sensations pulpal sensory neurites are important for regulating inflammatory reactions, blood flow, and dental issue repair (Byers and Narhi, 1999) (Heyeraas and Berggreen, 1999) (Hildebrand et al., 1995; Byers et al., 2003). The sympathetic nerve supply has a role in the regulation of blood flow and immune cell function (Olgart, 1996) (Haug and Heyeraas, 2006).

Periodontium innervated by sensory nerve fibers originating from the trigeminal ganglion (TG) and trigeminal mesencephalic nucleus (TMN) (Hildebrand et al., 1995) (Maeda et al., 1999). Nerves present in the periodontal space react to pressure, touch, position and injury. The free nerve endings emanating from the trigeminal ganglion (TMN) are primary nociceptive (A-delta and C-fibers) in nature, whereas Ruffini endings, originating from both TG and TMN are associated with periodontal fibers and are low-threshold mechanoreceptive receptors (Maeda et al., 1999) (Sato et al., 1988). Neurites originating from TMN are involved in proprioceptive control of mastication (Hildebrand et al., 1995) (Maeda et al., 1999). Sympathetic neurites are associated with arterioles in the periodontium (Hildebrand et al., 1995). Neurites in the periodontal space are also essential for hindering dento-alveolar ankylosis of tooth (Fujiyama et al., 2004). In the incisor periodontium, majority of the Ruffini and free nerve endings are present in the lingual side {Byers, 1989 #1022}(Naftel et al., 1999).

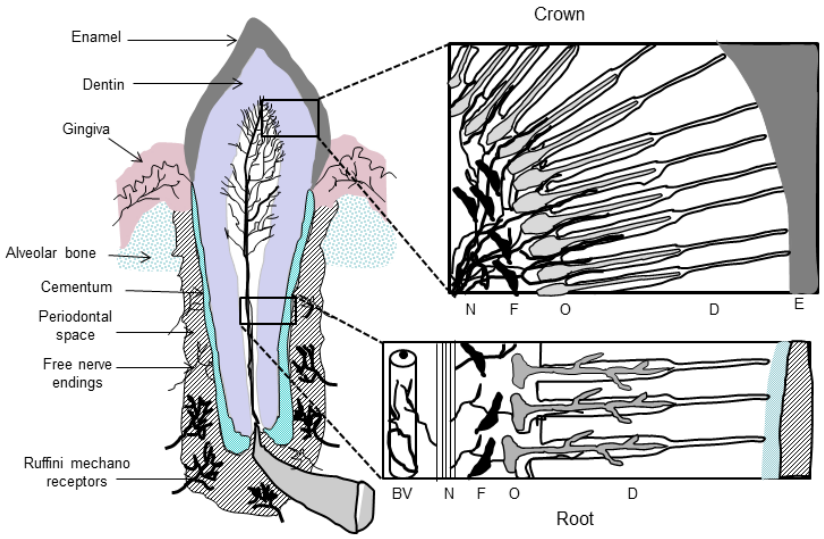

Figure 3. Schematic illustration of a mature erupted tooth and its dentinal, pulpal and periodontal innervation, with structures indicated. The higher-magnification panels show differences in distribution/pattern of neurites (N), pulpal fibroblasts (F) and

odontoblast morphology (0) for the crown and root. Vascular (BV) innervation is shown in the root diagram. Dentin (D), enamel (E). Modified from (Byers and Narhi, 1999).

1.4.1 Development of tooth nerve supply

The advancing tooth shape formation and cell differentiation is accompanied by development of the trigeminal innervation (Fig. 2) (Pearson, 1977) (Mohamed and Atkinson, 1983; Hildebrand et al., 1995) (Luukko et al., 2005a). In mouse, the development of the trigeminal ganglion starts around E9 (Davies et al., 1981). The first peripheral sensory neurites emerge half a day later and the last leave the ganglion at E13 (Davies et al., 1981). The growth rate is about 20 μm in hour (Davies, 1988). Sensory innervation of the tooth germ has been shown to take place later as compared to neighbouring tissues (Lumsden, 1982) (Mohamed and Atkinson, 1983). While the first trigeminal axons reach the mandibular developing skin epithelium at E10.5 (Davies, 1988), the pioneer "molar nerve" (Lumsden, 1982) emanating from the IAN is seen to course towards the mandibular first molar tooth germ at E12.5 (Luukko, 1997) (Kettunen et al., 2005). After reaching the tooth germ, the nerve divides into two branches, buccal and lingual one (Obara and Takeda, 1989) (Luukko, 1997) (Kettunen et al., 2005). The sensory innervation of the dental follicle target field commences at the cap stage (E14). The last trigeminal peripheral neurites have been reported to reach their target areas at around E15 (Davies, 1988) suggesting that the innervation of the dental-pulp target area is dependent on arborisation of the already existing nerves (Luukko et al., 2005a). Innervation of the coronal pulp starts when topography of the tooth crown has been established and a layer of dentin and enamel has been deposited in the pulp horns at around PN3-4 (Moe et al., 2008) (Mohamed and Atkinson, 1983) (Loes et al., 2002). In the mouse mandibular first molar, which has two roots, the mesial and distal one, the ingrowth of the neurites occurs specifically through the sites of the future roots (Luukko et al., 2008). Subsequently the main target area of the pulp, the coronal pulp-dentin border area, becomes innervated (Fig. 2) followed by the innervation of the radicular pulp and periodontal space as the root develops. Ingrowth of the sympathetic

neurites into the dental pulp of the mouse mandibular first molar appears to take place after the onset of root development at around postnatal day 9 (Moe et al., 2008).

1.5 Growth cone

Axonal growth and navigation is dependent on the motile structure at the distal tip of a growing neurite defined as a growth cone (Kolodkin and Tessier-Lavigne, 2011). The growth cone was first described by Santiago Ramon y Cajal (1890) as 'the expanded tips of growing nerve fibers (Puelles, 2009). It consists of three major components: filopodia, lamellipodia and the growth cone body (Fig. 4). Filopodia are long finger-like projections composed of long bundles of actin filaments. They participate in environment sensing and growth cone motility. Lamellipodia are veil-like protrusions commencing from the leading edge of growth cones consisting of a network of short, branched actin filaments and serve as the protrusion machinery of the growth cone (Vitriol and Zheng, 2012). The growth cone body, in turn, adheres to the suitable cells, resulting in a promotion and maintenance of growth cone navigation (Davies, 1997).

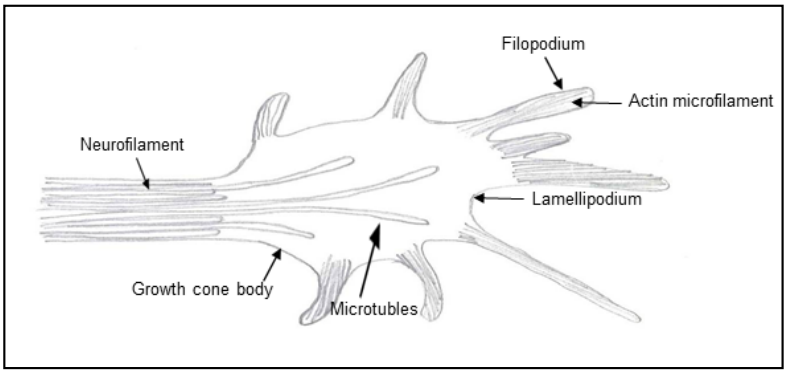

Figure 4. Schematic diagram of the growth cone. Modified from (Mueller, 1999).

1.6 Axon guidance molecules

Axon growth and navigation to the defined target area occur in a coordinated manner and is mediated by molecular interactions between the growth cone and extracellular

proteins and neighbouring cells (Huber et al., 2003). Growth cone expresses variety of proteins, many of which are receptors, which interact with their cognate ligands or interacting partners, which can act as guidance cues for growing axons (Bashaw and Klein, 2010) (Kolodkin and Tessier-Lavigne, 2011) (Tessier-Lavigne and Goodman, 1996). They appear to function principally by four mechanisms, such as contact repulsion, chemorepulsion, contact attraction and chemoattraction (Tessier-Lavigne and Goodman, 1996). Netrins, Slits, Semaphorins and Ephrins comprise four important axon guidance families (the "canonical cues") (Kolodkin, 1996) (Kolodkin and Tessier-Lavigne, 2011). Besides these molecules certain morphogens of Wnt, Hh and Tgf-beta signaling families are also known to be involved in neuronal guidance. Among the morphogens, axon-guidance functions of Wnt, Shh and Bmp in the innervation of spinal cord have been widely described (Kolodkin and Tessier-Lavigne, 2011) (Bovolenta, 2005). Cell-adhesion receptors such as immunoglobulin (Ig) cell-adhesion molecules (CAMs), cadherins and integrins have also been implicated in axon guidance (Huber et al., 2003). Cadherin superfamily members along with their role in fasciculation also regulate neuronal attraction (Kolodkin and Tessier-Lavigne, 2011). Integrins form a link between the cellular cytoskeleton and extracellular matrix by binding laminin, collagen and fibronectin as well as selected Ig CAMs (Huber et al., 2003). Some other secreted and chemotrophic factors such as Nerve growth factor (Ngf), Hepatocyte growth factor (Hgf), Insulin-like growth factor-1 (Igf-1) and Thrombin appears to act as axon guidance cues (Sanford et al., 2008). Endocytosis of receptor-ligand complex may be considered as an important mechanism, which regulates responsiveness of the growth cone to the guidance cue (Bashaw and Klein, 2010).

1.6.1 Semaphorins and their receptors

The semaphorins are a large family of secreted, transmembrane and GPI (glycosylphosphatidylinositol) -anchored proteins, which have a ~500 amino acid extracellular domain termed as a semaphorin domain in the N-terminal end. C terminus, which is a class-specific, may have additional sequence motifs. The name "semaphorin"

originates from the word “semaphore”, which refers to a long-distance communication system that is based on signaling. This family function as axonal guidance cues during neural development (Goodman et al., 1999) (Kolodkin et al., 1993) (Kolodkin, 1996). Semaphorins are subdivided into eight classes based on structural similarities (Kolodkin, 1996; Committee, 1999) (Mark et al., 1997) (Goodman et al., 1999). Class 1 and 2 semaphorins are present in invertebrates whereas class 3-7 are in the vertebrates and class V only in viruses. Classes 2, 3 and V semaphorins are secreted, and members of all other classes are transmembrane proteins (Fig. 5) (Committee, 1999; Tran et al., 2007).

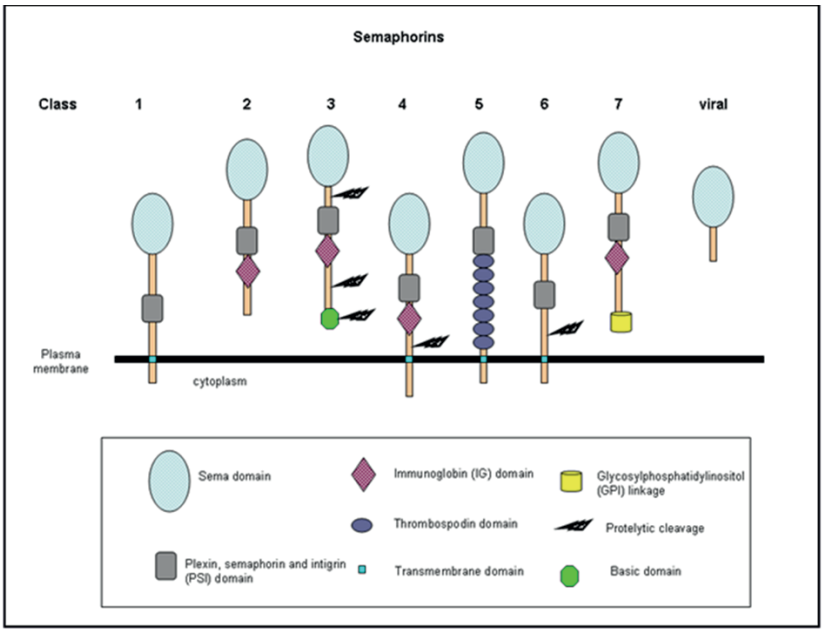

Figure 5. Schematic illustration of the semaphorin family. Modified from (Yazdani and Terman, 2006).

Semaphorins use as signal transducing receptors plexins to mediate cellular responses (Yazdani and Terman, 2006). Among all the classes of semaphorins, class 3 semaphorins (Sema3s) do not bind directly to plexins, but require neuropilin as a co-receptor for plexin based signaling function (Takahashi et al., 1999) (Raper, 2000) (Tamagnone and Comoglio, 2000) (Huber et al., 2003).

Plexins and neuropilins are type-1 transmembrane proteins (Tamagnone et al., 1999) (Kolodkin et al., 1997). Plexins are sub-classified into four groups: A, B, C and D (Kolodkin et al., 1997) of which class B Plexins undergo proteolytic processing in their extracellular part (Tran et al., 2007). Two neuropilins (Npn) have been characterized, namely Npn1 and Npn2 in mouse (Kolodkin et al., 1997). Semaphorins have been shown to serve neuronal and non-neuronal roles in development as well as in physiological and pathological processes of various organ systems (Kolodkin and Tessier-Lavigne, 2011). Similarly, plexins have been implicated in various biological functions such as innervation, vascular growth, epithelial tissue morphogenesis and tumour development (Tamagnone and Comoglio, 2004). Besides Plexins and neuropilins semaphorins interact with other receptors (Fig. 6) (Roth et al., 2009).

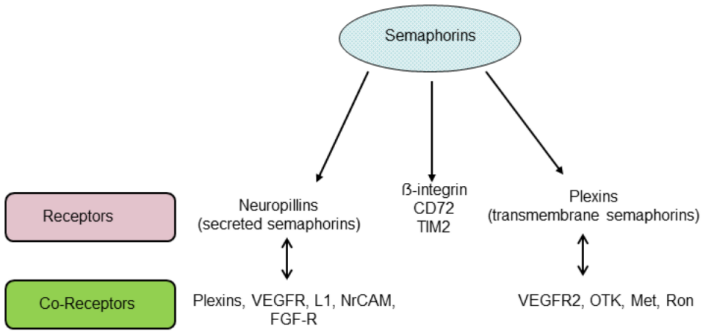

Figure 6. Semaphorin receptor and co-receptors (Roth et al., 2009).

Upon binding to their cell-membrane receptors semaphorin signaling has been shown to activate multiple intracellular signaling pathway such as MAP kinases, protein kinases and RhoGTPases as illustrated in the figure 7 (Roth et al., 2009).

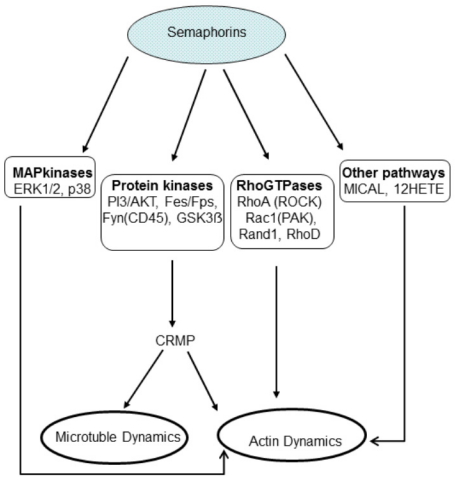

Figure 7. Semaphorin intracellular signaling cascades. Modified from (Roth et al., 2009).

1.6.2 Class 3 semaphorins and their receptors

The class 3 semaphorins (Sema3s) consist of seven members, Sema3A-3G (Yazdani and Terman, 2006). Molecular weight of class 3 semaphorin pro-protein is about 95 kDa. Proteolytic processing cleaves pro-protein into three peptides of 1, 33 and 65 kDa. Dimerization of sema3s is necessary for their function (Klostermann et al., 1998) (Adams et al., 1997; Koppel and Raper, 1998). During development, class 3 semaphorins are broadly expressed in neuronal as well as non-neuronal such as the skeletal, cardiovascular, gastrointestinal, renal, and respiratory tissues (Kruger et al., 2005) (Yazdani and Terman, 2006).

Sema3s function as secreted chemo-repulsive cues for specific types of peripheral and central nervous systems neurons, but some members can act as either repellents and/or attractants depending on their spatial distribution and the receptor complex combination in target cells (Bagnard et al., 1998) (Gu et al., 2005a) (Chauvet et al., 2007). The first characterised semaphorin in vertebrates was Sema3A. It was found to induce collapse of growth cones in dorsal root and sympathetic ganglion neurites *in vitro* (Luo et al., 1993)

(Koppel et al., 1997). Later Sema3A was found to be a selective chemorepellent for several subsets of neurites such as Ngf but not Nt3-responsive DRG nerve fibers, as well as geniculate, trigeminal, olfactory, sympathetic and most cranial motor and hippocampal neurites *in vitro* (Messersmith et al., 1995) (Puschel et al., 1995) (Vilbig et al., 2004) (Dillon et al., 2004) (Chedotal et al., 1998) (Varela-Echavarria and Guthrie, 1997) (Kobayashi et al., 1997) (Adams et al., 1997). Moreover, Sema3A repels cortical neurites and inhibits their branching (Bagnard et al., 1998) (Dent et al., 2004).

Genetic investigations have shown that sema3s serve essential both neuronal and non-neuronal functions *in vivo*. Mice deficient for Sema3A display severe abnormalities in peripheral nerve projections of cranial nerves including trigeminal, facial, vagus, accessory, and glossopharyngeal nerves (Taniguchi et al., 1997) (Ulupinar et al., 1999) (Rochlin et al., 2000). In addition, patterning of spinal nerves is abnormal and sympathetic neurons and neurites are defasciculated (Taniguchi et al., 1997). Sema3A also serves important roles in orientation of neuronal processes in the cortex as well as in organogenesis of heart and skeletal system (Behar et al., 1996) (Taniguchi et al., 1997). Recently Sema3A signaling was found to regulates bone mass by affecting to osteoclast and osteoblast activity (Hayashi et al., 2012). Sema3B is needed for a proper positioning of the anterior commissure in CNS (Falk et al., 2005). In addition, it stimulates osteoclastogenesis and induces osteopenia (Sutton et al., 2008). Sema3C null mice die soon after birth due to congenital cardiovascular defects (Feiner et al., 2001). Sema3D serves a role in the patterning of pulmonary veins (Degenhardt et al., 2013) and regulate branching of peripheral neurites in zebrafish (Liu and Halloran, 2005). Sema3E transcripts are expressed in developing somites and regulates pattering of the intersomitic blood vessels by acting as repellent for plexin-D1-expressing endothelial cells (Gu et al., 2005a). In developing CNS, Sema3E acts as repellent for neurons expressing PlexinD1 and attractant for those expressing both PlexinD1 and neuropilin-1 (Chauvet et al., 2007). It also promotes elongation of axons expressing Vegfr2, PlexinD1 and Npn1 in brain (Bellon et al., 2010). Sema3F is necessary for fasciculation of anterior commissure and decussation, development of limbic system and amygdaloid circuitry as well as patterning and fasciculation of specific peripheral nerves (Sahay et

al., 2003 {Cloutier, 2004 #965) (Huber et al., 2005). Endothelial cell expressed Sema3G may regulate angiogenesis but its functions, if any, appear to be redundant (Kutschera et al., 2011).

In vitro experiments have provided further support for non-neuronal roles for sema3s signaling during organogenesis for example in the development of budding organs such as lung, salivary glands and kidneys. Sema3A inhibits whereas Sema3C and -3F stimulate embryonic lung branching morphogenesis (Ito et al., 2000) (Kagoshima and Ito, 2001). Sema3A and -3C additively stimulate cleft formation in developing submandibular salivary gland (Chung et al., 2007). In kidney, Sema3A is a negative regulator of ureteric bud (Tufro et al., 2008) and regulates expression of *Plexin-A1-A3* and *-D1* in immortalized glomerular podocytes (Guan et al., 2006).

Plexin-A1-A4 (Plexin-As) and -D1 serve as main class 3 semaphorin signal transducing receptors. Plexin-As have a molecular weight of about 220 kDa (Maestrini et al., 1996) (Tamagnone et al., 1999). With exception of Sema3E, class 3 semaphorins also require ligand binding co-receptor, neuropilin to form signal transducing holoreceptor complex (Yaron et al., 2005) (Fig. 8). Two neuropilins, namely Npn1 and -2, which have a molecular weight of about 130 kDa have been reported in mouse (He and Tessier-Lavigne, 1997) (Kolodkin et al., 1997). In addition, plexin-B1, L1cam and Chl1 act as class 3 semaphorin receptors (Sharma et al., 2012).

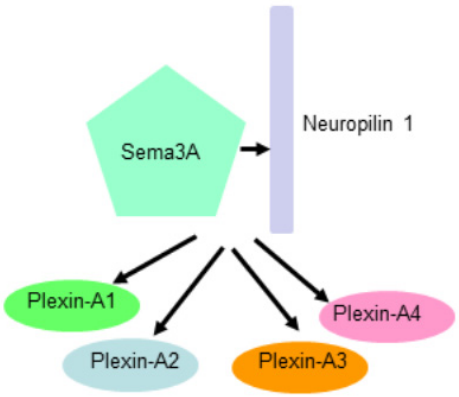

Figure 8. Sema3A interactions with Plexin-A (A1-A4) and Neuropilin-1 receptors. Modified from {Sharma, 2012 #1059}.

Class 3 semaphorin receptors have been reported in many tissues during embryonic and postnatal development. Neuropilins are widely expressed in both neuronal and nonneuronal tissues (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) Similarly, plexin-A1-A4 mRNAs are broadly expressed in the developing central nervous system (Perala et al., 2005). Sensory and sympathetic ganglia of the peripheral nervous system express *plexin-A1-A4*. Their expression in non-neuronal tissues is more distinct. Whereas *Plexin-A1* and *-A2* mRNAs have been reported in various non-neuronal organs, *plexin-A3* and *-A4* show more limited expression (Tamagnone et al., 1999) (Perala et al., 2005). *PlxnD1* has been reported to be expressed the embryonic central nervous systems and vascular endothelium of different tissues (van der Zwaag et al., 2002) (Chauvet et al., 2007).

Genetic and *in vitro* experiments have provided increasing evidence that Plexins and neuropilins mediate class 3 semaphorin signaling *in vivo*. For instance, PlexinA1 is involved in bone homestasis and immunoresponse (Takegahara et al., 2006). PlexinA2 signaling regulates functions of cerebellar cranule cells (Renaud et al., 2008). Differential signaling of plexin-A3 and -A4 as well as Npn1 and -2 mediate effects of class three semaphorins, Sema3A and -3F, in sensory and sympathetic neurons (Cheng et al., 2001) (Yaron et al., 2005) (Schwarz et al., 2008). Sema3A-Npn-1 and Sema3F-Npn-2 signaling control motor axon growth and guidance (Huber et al., 2005). Npn1/Sema3E/PlexinD1 signaling control neuronal connectivity in CNS (Chauvet et al., 2007). Npn1/Sema3E/PlexinD1 signalling regulates cardiovascular development (Gitler et al., 2004) (Torres-Vázquez et al., 2004) (Gu et al., 2005b). PlexinD1 is needed for skeletal morphogenesis (Kanda et al., 2007).

1.6.3 Class 4 Semaphorins and their receptors

The class 4 semaphorins (sema4s), namely Sema4A-4G, are type I transmembrane proteins, and have a molecular weight of about 150 kDa. Sema4D (CD100) was identified in 1992 in T-lymphocytes (Bougeret et al., 1992). Sema4s form 300 kDa homodimers, which undergo proteolytic processing in the extracellular part (Tran et al., 2007). Due to cleavage Sema4D exists also as a soluble 240-kDa homodimer in the lymphatic tissue (Elhabazi et al., 2001) (Nkyimbeng-Takwi and Chapoval, 2011) (Delaire et al., 2001). Sema4E has been identified in zebrafish (Yazdani and Terman, 2006).

Like other semaphorins, sema4s are involved in various biological activities including immune response, neuronal development and angiogenesis (Yazdani and Terman, 2006). Transcripts of Sema4s show broad, partially overlapping expression domains in the developing nervous system and non-neuronal tissues and their signaling serves essential functions, some of which are briefly mentioned below. Sema4A and Sema4B transcripts have been reported in the olfactory epithelium (Williams-Hogarth et al., 2000). Sema4C mRNAs show expression in the developing CNS, trigeminal ganglion, DRG, olfactory epithelium, E16 tooth germ, lung and kidney (Inagaki et al., 1995) (Williams-Hogarth et al., 2000). Besides being expressed in the CNS and trigeminal and dorsal root ganglion, Sema4D mRNAs have been reported in developing tooth at E17 as well as DRG, kidney, olfactory epithelium and lung (Worzfeld et al., 2004) (Fazzari et al., 2007) (Korostylev et al., 2008). In addition to developing nervous system, branchial arches display *Sema4E* expression in zebrafish (Xiao et al., 2003). *Sema4F* is expressed in the spinal motor neurons, DRG, sympathetic ganglia as well as retinal ganglion cells (Encinas et al., 1999). Sema4G have been observed in developing brain, spinal cord, trigeminal ganglion, DRG, several sensory organs as well as kidney, liver and gut (Li et al., 1999).

The neuroimmune semaphorin Sema4A serves critical regulatory roles in immunosystem (Kumanogoh et al., 2005) and appears to suppress angiogenesis via Plexin-D1 (Toyofuku et al., 2007). In addition, Sema4A-deficient mice display retinal

degeneration (Rice et al., 2004). Sema4B is involved in basophil mediated immunoresponses (Nakagawa et al., 2011). Sema4C and Sema4G act as ligands in PlexinB2 signaling mediated cerebellar development (Maier et al., 2011). Another neuroimmune semaphoring, Sema4D serves crucial functions in the immune response (Kumanogoh and Kikutani, 2013) (Nkyimbeng-Takwi and Chapoval, 2011). Sema4D also act as a collapsing factor on hippocampal and retinal axonal growth cones in culture (Masuda et al., 2004) (Ito et al., 2006) (Swiercz et al., 2002), and enhances neurite outgrowth from the embryonic cortical but not from the DRG explants (Worzfeld et al., 2004). Recently, osteoclast expressed Sema4D was reported to suppress bone formation (Negishi-Koga et al., 2011). Sema4E act as an axon guidance cue to facial and gill motor neurites, and contribute to their fasciculation in zebrafish (Xiao et al., 2003).

Plexin-B1, -B2 and -B3 as well as -D1 mediate sema4s signalling (Fig. 9) (Yukawa et al., 2010). *PlexinB1* and *-B2* display largely similar expression domains in the nervous system and non-neuronal tissues during embryogenesis (Worzfeld et al., 2004) (Perala et al., 2005) (Korostylev et al., 2008). *Plexin-B1,* but not *Plexin-B2,* is reported in both trigeminal and dorsal root ganglia (Perala et al., 2005). Plexin-B3 receptor mRNAs are exclusively detected in the postnatal oligodendrocytes (Worzfeld et al., 2004) and Plexin-B3-deficent mice show no apparent abnormalities (Worzfeld et al., 2009). CD72 and Tim-2 (T cell immunoglobulin and mucin-domain-containing 2) act as Sema4D and Sema4A receptors, respectively, in immune cells (Kumanogoh et al., 2000) (Kumanogoh et al., 2002).

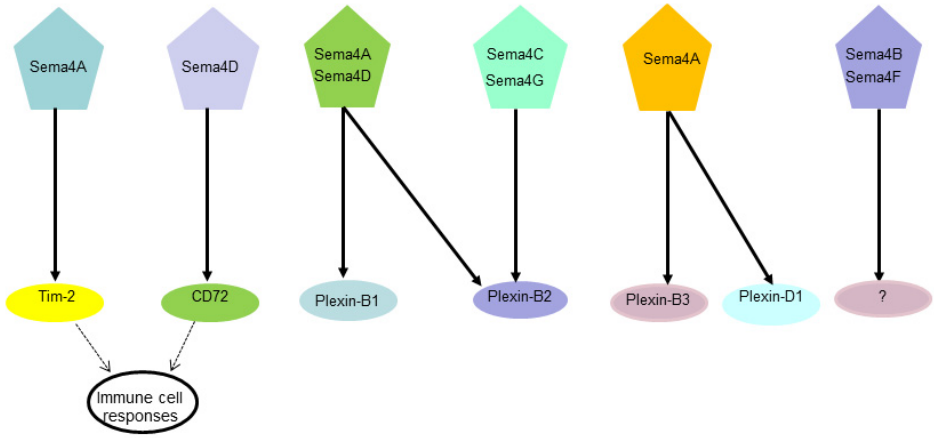

Figure 9. Class 4 semaphorins and receptors. Sema4A interacts with plexins (-B1, -B2, -B3 and D1) and Tim-2 receptors. Sema4D binds plexin-B1, plexin-B2 and CD72 receptors. Modified from (Zhou et al., 2008; Yukawa et al., 2010).

1.7 Molecular control of tooth sensory innervation

Studies with various species in particular mouse and rat have revealed that neurite pathfinding to the developing tooth which is a unique, specialized target organ, is developmentally regulated and tightly associated with progressing odontogenesis (Pearson, 1977) (Mohamed and Atkinson, 1983; Hildebrand et al., 1995) (Luukko, 1997) (Kettunen et al., 2005) (Luukko et al., 2008). This has suggested that tooth innervation is dependent on tooth-specific regulation.

Peripheral axon pathfinding and subsequent innervation of the various target tissues are regulated by different diffusible and contact-based neuroregulatory molecule families, which exert positive and negative influence on growing axons (Tessier-Lavigne and Goodman, 1996). There is increasing body of and molecular, experimental and genetic data indicating that also the innervation of the tooth is regulated by molecules of different families guiding the development of the nervous system in general (Fried et al.,

2000) (Nosrat et al., 1998) (Fried et al., 2007) (Luukko et al., 2005a) (Luukko and Kettunen, 2014).

Ngf-related neurotrophic growth factors, collectively referred to as neurotrophins (Lewin and Barde, 1996), and glial cell line–derived neurotrophic factor (Gdnf)-family are important regulators of the innervation of the peripheral organs, and members of both families have been reported in the developing tooth. In particular, *Ngf* and *Gdnf* show developmentally regulated cellular expression patterns in both embryonic and postnatal molars that correlate with the dental neurite growth and innervation of the dental target fields (Byers et al., 1992) (Mitsiadis et al., 1993) (Mitsiadis et al., 1992) (Luukko et al., 1998) (Luukko et al., 1997b) (Luukko et al., 1997a) (Nosrat et al., 1997). Ngf mRNAs are specifically expressed in the mesenchymal trigeminal axon pathway of the molar tooth germ during pioneer dental axon growth (Luukko et al., 1997a). Subsequently, *Ngf* and *Gdnf* are co-localized in the dental follicle target field. Postnatally, both of them show distinctive expression in the odontoblast and subodontoblastic region of the important dentin-pulp border target area (Luukko et al., 1997b) (Luukko et al., 1997a) (Nosrat et al., 1997) (Nosrat et al., 1998). In addition, Ngf-immunoreactivity has been reported in the odontoblasts (Woodnutt et al., 2000). Ngf signaling is essential for the dental sensory and sympathetic innervation as demonstrated using the tyrosine kinase A (trkA) receptor-deficient mice, which show absence of sensory and sympathetic innervation in the pulp and a reduced innervation of the periodontium (Matsuo et al., 2001). In addition, mice-deficient for p75 showed fewer nerve endings in the incisor periodontium and increased CGRP levels in the molar pulp (Sarram et al., 1997). Exposure of NGF antibodies resulted in reduced number of sensory axons in the postnatal dental pulp (Qian and Naftel, 1996) (Naftel et al., 1994). Analysis of transgenic mice has indicated that Neurotrophin-4/5 (Nt-4/5), Brain derived neurotrophic factor (Bdnf) and trkB signaling control the development and/or maturation of the periodontal Ruffini endings (Alkhamrah et al., 2003) (Maruyama et al., 2005) (Matsuo et al., 2002).

Members of different semaphorin families show dynamic mRNA expression in the developing tooth suggesting a role in tooth innervation (Loes et al., 2001) (Lillesaar and Fried, 2004) (Sijaona et al., 2012). In particular, *Sema3A* is dynamically expressed in the embryonic and postnatal restriction areas of pre- and postnatal molar tooth germ suggesting an important role in the development of tooth nerve supply (Loes et al., 2001; Kettunen et al., 2005; Luukko et al., 2008). Sema3A-deficient molar tooth germs become prematurely innervated and the apparently defasciculated neurites exhibit patterning defects demonstrating that Sema3A acts as a local, developing tooth target produced signal, which regulates timing and patterning of molat tooth innervation (Kettunen et al., 2005) acting apparently partly through neuropilin-1 (Npn1) (Kettunen et al., 2005) (Kitsukawa et al., 1997). In the postnatal mouse mandibular first molar, Sema3A is specifically located in the middle area of the base of the pulp and circulates the future mesial and distal root areas suggesting a function in the dental pulp innervation (Kettunen et al., 2005; Luukko et al., 2008). That Sema3A and neuropilin-1-deficient embryos show apparent abnormalities in the sympathetic neurons and sympathetic trunk (Taniguchi et al., 1997) (Kawasaki et al., 2002) suggests that repulsive Sema3A signaling regulates development of tooth sympathetic nerve supply as well. In contrast, odontoblasts expressed Sema7A has been shown to promote trigeminal axon growth *in vitro* and may therefore regulate terminal innervation of the dentin-pulp complex target area (Maurin et al., 2004).

Besides Sema3A, other class three Semaphorins, Sema3B, -C, -E, and -F and *Npn-1* and *-2* as well as *Plexin-A4* and *-A3* receptor mRNAs have also been reported to exhibit dynamic expressions in the developing tooth using sectional *in situ* hybridization and/or PCR (Loes et al., 2001) (Lillesaar and Fried, 2004) (Luukko et al., 2005a) (Perala et al., 2005) (Sijaona et al., 2012). In addition, their signaling receptors, *Npn-1* and *-2* as well as *Plexin-A4* and *-A3* were reported in the trigeminal ganglion cells during tooth development (Kettunen et al., 2005; Sijaona et al., 2012). In the developing mouse tooth, Plexin-B1 receptors were seen in the dental epithelium (Korostylev et al., 2008; Perala et al., 2011). Overexpression of Sema4D may inhibit odontoblast differentiation (Abe et al., 2008). RT-PCR study of expression of semaphorins in the dental papilla and pulp

mesenchyme during embryonic and postnatal stages revealed that mRNA levels of Sema4A were lower during embryonic stages compared to postnatal stages whereas expression of *Sema4B, -4C, -4D* and *-4G* remained fairly constant or varied (Lillesaar and Fried, 2004).

Expression of molecules belonging to other canonical guidance cues Netrin, Slit, and Ephrin families has been reported in the developing tooth suggesting neuroregulatory functions (Loes et al., 2003) (Luukko et al., 2005b) (Loes et al., 2001). In addition, other molecules such as Ncam, integrins, laminins and reelin may serve important roles in tooth innervation (Obara and Takeda, 1993) (Maurin et al., 2004) (Fried et al., 2005) (Fried et al., 2000) (Fried et al., 2007). It is also possible that morphogens and growth factors (Kolodkin and Tessier-Lavigne, 2011) regulating odontogenesis serve important roles in tooth innervation. Indeed, conserved Wnt, Fgf and Tgfß-families were shown to be involved in regulation of *Sema3A* expression in developing tooth (see below) (Kettunen et al., 2005) (Kettunen et al., 2007).

1.8 Tissue interactions in tooth innervation

The findings that tooth innervation is tightly linked with advancing histomorphogenesis of the tooth, and that many of the molecules implicated in regulation of tooth innervation show developmentally regulated cellular expression domains that correlate with dental axon growth and pattering have suggested that the developing tooth germ is involved in regulation of the development of its own nerve supply. This hypothesis has received support from various experimental and genetic experiments. Replanted tooth anlages show ability to promote it own innervation and some re-innervation in denervated or reimplanted adult teeth has been found to take place (Erdelyi et al., 1987) (Fried and Erdelyi, 1982) (Holland and Robinson, 1987) (Holland and Robinson, 1985). Whereas *in vitro* cultured early dental mesenchyme explants were found to repel trigeminal neurite growth, late prenatal and early postnatal mesenchymes attracted nerve fibers (Lillesaar et al., 1999) (Lillesaar and Fried, 2004). In addition, expression

of Ngf and Nt-3 mRNAs in cultured tooth anlages was observed to be not dependent on or regulated by peripheral neurites (Lallier, 2004).

Heterotypic interactions, which take place between the epithelial and mesenchymal cells, are considered to constitute the single most important mechanism regulating vertebrate organ development (Gurdon, 1992). Investigations of the regulation of *Sema3A* have provided evidence that epithelial-mesenchymal interactions are involved in regulation of tooth innervation (Kettunen et al., 2005). Early dental epithelium and Wnt4 and Tgfß1 signals expressed in the early dental epithelium, are capable to induce *Sema3A* expression in the dental mesenchyme (Kettunen et al., 2005). Thus, epithelial-mesenchymal interactions control *Sema3A* expression and are proposed to coordinate peripheral dental axon navigation and patterning with tooth formation. Presumptive/early dental epithelium expressed Wnt4 was also shown to regulate expression of *Msx1* transcription factor, and Tgfß1 dental mesenchyme proliferation (Kettunen et al., 2005). Mice deficient for the dental epithelium expressed Fgfr2b, which mediate odontogenic epithelial-mesenchymal signaling (Kettunen et al., 2000) (Harada et al., 1999) (Yokohama-Tamaki et al., 2006), showed downregulation of *Fgf4* and *Tgfß1* in the molar tooth germs as well as exhibited defects in the mesenchymal expression domains of *Sema3A* and dental neurite navigation and patterning (Kettunen et al., 2007). Moreover, the primary and secondary enamel knot expressed Fgf4 was found to regulate mesenchymal *Tgfß1* expression (Kettunen et al., 2007). Collectively these data provided evidence that local tissue interactions, mediated by conserved signals, regulate both tooth morphogenesis and dental sensory innervation. It has been proposed that the integration of the local innervation-regulating signaling pathways with large signaling networks regulating tooth formation of tooth germ proper constitutes the key mechanism whereby tooth innervation is spatiotemporally coordinated with advancing odontogenesis (Luukko et al., 2005a). The observation that signaling from the presumptive dental epithelium controls Sema3A in the underlying mesenchyme suggests that the early dental epithelium possesses the odontogenic information to regulate both tooth formation (Mina and Kollar, 1987) (Lumsden, 1988) and tooth-specific nerve supply (Kettunen et al., 2005).

2. OBJECTIVES

The hypothesis of the study states that Sema3A is expressed in the developing mouse incisor and that Sema3A has neuronal and non-neuronal functions in developing incisor and postnatal molar and that class 4 semaphorins are expressed in mandibular first molar tooth germ during early postnatal development and have neuronal and non-neuronal functions.

Main goal

The main goal of this study was to address putative roles of Sema3A and class 4 semaphorins and their receptors in odontogenesis.

Secondary goals

-To analyse the expression patterns of *Sema3A* in the embryonic and postnatal mouse mandibular incisor tooth germ
-To study functions of Sema3A in embryonic and postnatal developing incisor as well as postnatal molar tooth innervation using Sema3A-deficient mice
-To investigate mRNA expression of neuroregulatory molecules Ngf, Gdnf and Ncam in postnatal Sema3A-deficient mouse incisor and molar as well as their receptors trkA, p75 and Ret, Npn-1, Plexin-A4 in the postnatal mouse Sema3A-deficient trigeminal ganglia
-To study the histo-morphology of the Sema3A-deficient incisor tooth germ, adult incisor and postnatal molar
-To analyse mRNA expression patterns of Sema4A-4D, -4F and -4G as well as their receptors PlexinB1 and -B2 in the early postnatal developing mouse mandibular molar tooth germ as well as presence of protein products of Sema4D, PlexinB1 and -B2 in postnatal trigeminal ganglion

3. MATERIALS AND METHODS

3.1 Animal material and tissue preparation

3.1.1 Mouse husbandry

The use of animals in this project was approved by the Norwegian Animal Research Authority and animal experiments were carried out under ethical standards approved by the Norwegian Committee for Experiments on Animals and EU directive 2010/63/EU. The animal room had a controlled temperature (22^{o}C), humidity (around 41%) and ventilation (18 air changes/h). The room was kept on a regular 12-h light/12-h dark cycle. Mice were housed in wire-topped Scanbur (Scanbur, Karlslunde, Denmark) polycarbonate cages provided with aspen bedding in a paper bag, which mice used as nesting material. As an environmental enrichment to increase welfare and to alleviate stress, transparent red Mouse Igloos, Mouse Houses and aspen bricks (Scanbur, Karlslunde, Denmark) were introduced into the cages. Females were socially housed with 2-6 individuals kept together in a cage. Males were also housed socially (up to 6 males in a cage) prior to them being used for breeding. After the first breeding each male had its own cage. Mice were given free access to drinking water and standard pellet food (Rat and Mouse No.1 Maintenance diet, Special Diets Services, Essex, UK, product code 801002), but mice in breeding cages and females with nursing pups up to an age of three-to-four weeks were fed a more nutritious pellet diet (Rat and Mouse No. 3 Breeding diet, Special Diets Services, product code 801066).

To obtain mouse embryos and foetuses of a known gestational age, one NMRI (Naval medical research institute, USA) (outbred stock) male together with 1-3 female mice were kept in each breeding cage over three nights and female mice were checked for copulation plugs every morning. The day of finding a copulation plug was counted as embryonic day 0 (E0) and was further confirmed by morphological criteria according to Theiler, 1989. The date of birth was designated as postnatal day 0 (PN0). Tissues were collected on embryonic (E) days 11.5, 12.5, 13.5, 14.5, 16.5 and 18.5 and postnatal days

0, 1, 2, 5 and 7. On average mice from three litters were examined at each stage. The embryos and pups were euthanized by decapitation. The heads of embryos and mandibles of postnatal pups were immersion fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight with gentle shaking, decalcified in ethylenediaminetetraacetic acid (EDTA) and embedded in Paraplast (McCormick Scientific) or Gurr (BDH Prolab) paraffin. Serial frontal (E11.5, 12.5, 13.5, 14.5, 16.5 and 18.5), and sagittal (PN0, 1, 2, 3, 5 and 7) sections of 7 µm were cut using a Leica RM 2235 microtome (Leica Microsystems, Wetzlar, Germany) for *in situ* hybridisation.

Two different strains of Sema3A deficient mice of C57BL/6 and CD1 background were analysed (Taniguchi et al., 1997). Timed breeding of *Sema3A*$^{+/-}$ mice was performed as described for NMRI mice above. Pups from PN0, 1, 2, 3, 4 and 7 and adult (three months old) stages were collected. Adult mandibles were immersion fixed in 4% PFA for 7 hours and demineralized in 10% EDTA for 10 days. They were cut into 50 µm thick floating sections and stored in PBS solution at 4°C until used. The total number of mice utilized in the whole project was 400.

3.2 Genotyping of mice (Articles I-III)

Genotyping of the mice was performed using polymerase chain reaction (PCR) as described previously (Taniguchi et al., 1997). Ear pieces from mice were used as genomic DNA source. A very small (1.5-2 mm in diameter) ear piece was collected when each mouse was earmarked for individual recognition. Genomic DNA was isolated from each tissue sample using Promega Wizard genomic DNA purification kit (A1620) according to manufacturer´s protocol. PCR was performed using GoTaq DNA polymerase (Promega, M3001), primers, water, PCR buffer, $MgCl_2$, nucleotide mix and a genomic DNA template. Sema3A mice in C57BL/6 background were genotyped with the following primers: 5'-ACAACGCTTGCCTCGGGAGGTAAA-3', and 5'-ATGGTTCTG ATAGGTGAGGCATGG-3' (Taniguchi et al., 1997). PCR was performed at 96°C for 5 min, 75°C for 5 min, 60°C for 3min, and 75°C for 5 min, and thereafter at 96°C for 30 s, 60°C for 30 s, and 72°C for 2 min for 35 cycles. Sema3A

mice in CD1 background were genotyped with a primer pair 5'-GTTCTGCTCCCGGCTCTAAATCTC-3' and 5'-ATGGTTCTGATAGGTGAGGCATGG-3'). PCR was performed at 96^{o} C for 5min, 75 ^{o} C for 5 min, 55 ^{o} C for 3 min and thereafter at 96^{o} C for 30 seconds, 60^{o} C for 30 seconds, and 72^{o} C for 2 min for 35 cycles.

3.3 Antibodies

The primary and secondary antibodies used in the study are displayed in Table 1.

Table 1. The antibodies used in the study

Primary antibody	Type	Company	Dilution	Secondary antibody*	Method
Rabbit polyclonal anti rat Peripherin	IgG	Millipore (Chemicon International) CA, USA AB1530	1:250	Biotinylated goat anti-rabbit 111-066-006	IHC
			1:500	FITC conjugated goat anti-rabbit 111-095-144	IF
			1:500	Cy3 conjugated donkey anti- rabbit 711-165-152	Thick tissue confocal imaging
Mouse monoclonal Anti-neurofilamen	IgG1	Sigma-Aldrich, MO, USA N0142	1:500	Rhodamine conjugated Affinipure donkey anti-mouse 715-295-151	IF

t 200					
Rabbit polyclonal anti-Plexin-B1 (H-300)	IgG	Santa Cruz Biotechnology, Texas, USA sc-25642	1:200	Horseradish peroxidase conjugated goat anti- rabbit 32460	WB
			1:100	Biotinylated goat anti- rabbit 111-066-006	IHC
Sheep polyclonal Anti-human Plexin-B2	IgG	R&D Systems, MN, USA AF5329	1:40	Horseradish peroxidase conjugated rabbit anti-sheep 313-035-003	WB
			1:20	Biotinylated donkey anti- sheep 713-065-003	IHC
Goat polyclonal anti-Sema4D (CD100) (Y-20)	IgG	Santa Cruz Biotechnology, Texas, USA sc-79405	1:200	Horseradish peroxidase conjugated rabbit anti- goat. Dako cytomation, Glostrup,Denmark, P0160	WB
			1:100	Biotinylated donkey anti-goat. 705-066-147	IHC

IHC: Immunohistochemistry; IF: Immunofluorescence; WB: Western Blot.

*All biotinylated, FITC, Cy3, Rhodhamine and peroxidase – conjugated secondary antibodies (except the company specified in the table) were obtained from Jackson ImmunoResearch Laboratories, West Grove, PA, USA.

3.4 Immunohistochemistry and immunofluorescence (Articles II and III)

Trigeminal ganglia were collected from PN2, PN5 and PN7 NMRI mouse pups. The trigeminal ganglia were fixed in 4% PFA overnight, embedded in Tissue-Tek OCT (Sakura Finetek Europe B.V., Alphen and den Rijn, Netherlands) and cut into 20µm thick sections using a Leica RM 2235 cryotome (Leica Microsystems, Wetzlar, Germany). Microwave antigen retrieval was performed with 1% citrate buffer at 750W for 10 min. Immunohistochemistry was carried out using Rabbit polyclonal anti-human Plexin-B1 antibody (Santa Cruz Biotechnology, Texas, USA; 1:100 dilution), Sheep polyclonal anti-human Plexin-B2 (R&D Systems, MN, USA; 1:20 dilution) and Goat polyclonal anti-human Sema4D (CD100) antibody (Santa Cruz Biotechnology, Texas, USA; 1:100 dilution) and biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA) at a dilution of 1:500 were used. The Avidin-biotin peroxidase complex method (PK-6100, VECTASTAIN Elite ABC Kit, Vector Laboratories, CA, USA) was performed according to the instructions of the manufacturer using 3-amino-9-ethylcarbazole (AEC) (A6926, Sigma-Aldrich, Inc, MO, USA) as a colour substrate. The primary antibody was omitted in the control sections and no specific staining was seen.

Fresh frozen tissues of *Sema3A*$^{+/+}$, *Sema3A*$^{+/-}$ and *Sema3A*$^{-/-}$ mice were embedded in Tissue-Tek OCT (Sakura Finetek Europe B.V., The Netherlands). At least two sets of serial frontal frozen sections of first molars and incisors (30 µm thickness) per genotype per age were examined. Frozen sections were fixed with 4% PFA. In order to stain sensory nerve fibers in the dental papilla and pulp polyclonal rabbit anti-rat peripherin antibody (AB1530, Chemicon international, CA, USA; 1:250 dilution) was used as primary antibody and goat anti rabbit antibody (Pierce 1858415, Jackson Immunoresearch laboratory, PA, USA) was used as a secondary antibody. Antigen

retrieval was performed with 0.05% Trypsin (Sigma-Aldrich, Inc, MO, USA) for 3 minutes at 37°C. Sections were washed in PBS and treated with 3% hydrogen peroxide in 100% methanol for 30 minutes at 4°C. After PBS washes, the sections were incubated with normal goat serum for 30 min at room temperature. Thereafter, the serum was drained off and incubation with primary antibody in 0.2% diluted in PBS/BSA for 24 hours took place. The sections were rinsed with PBS, incubated with secondary antibody for 60 min and subsequently rinsed in PBS. Immunohistochemistry on adult floating sections was carried out using rabbit polyclonal affinity purified anti-UCHL1 (PGP 9.5) antibody (1:2000 dilution) (HPA005993, Sigma-Aldrich, Inc, USA). Avidin-biotin peroxidase complex technique was used at 37ºC for 30 min using Vectastain Elite ABC kit (PK-6100; Vector laboratories, Burlingame, CA, USA) according to manufacturer's instructions and 3-Amino-9-ethyl-carbozole (AEC) (Sigma–Aldrich, Inc., MO, USA, A6926) was used as colour substrate. No specific staining was seen in control sections.

In this study indirect double immunofluorescence staining was used to compare the sensitivity of the polyclonal mouse anti-Neurofilament 200 (Clone N52; N0142, Sigma-Aldrich, Inc, MO, USA; 1:250 dilution) and rabbit monoclonal anti-rat Peripherin (AB1530, Chemicon international, CA, USA) antibodies to detect trigeminal nerve fibers in PN3 dental pulp. FITC-conjugated goat anti rabbit IgG (H+L) (Jackson ImmunoResearch; 111-095-144; 1:500 dilution) and Rhodamine Red-X-conjugated F(ab)2 donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch; 715-295-151; 1:500 dilution) for Peripherin and Neurofilament 200, respectively, were used as secondary antibodies. Immunofluorescence immunostaining was performed on 30 µm thick fresh frozen sections.

3.5 Thick tissue confocal imaging (Articles II and III)

In order to trace the course and ramification of sensory nerve fibers into the pulp-dentin border area, which is the final target area in the dental pulp, floating section immunohistochemistry was performed. PN7 mandibles from Sema3A$^{-/-}$ and Sema3A$^{+/+}$

mice were cut into 100 µm thick sections and stained with rabbit polyclonal anti-Peripherin antibody (AB1530, Chemicon international, CA, USA). Cy3-conjugated donkey anti rabbit IgG (H+L) antibody (Jackson ImmunoResearch 711-165-152; 1:250 dilution) was used as secondary antibody. Subsequently, sections were cleared with BABB (benzyl alcohol/benzyl benzoate; Sigma-Aldrich; St. Louis, MO) solution and placed on the cavity slide in the mounting medium Immu-mount. Imaging was executed using a Leica SP5 AOBS confocal microscope equipped with a 561 nm DPSS laser. Two optical stacks were needed to cover the whole area of the incisor and molar and they were stitched using Xuvtools (Emmenlauer et al., 2009). Optical sections were volume-rendered with the maximum intensity projection method using Imaris software (Bitplane, Switzerland). Volume- rendered images were animated using Imaris software (Bitplane, Switzerland) and further processed with Quicktime Pro software (Apple Inc, USA). Snapshots of volume-rendered images were used to make an image plate with Adobe Photoshop CS4 software.

3.6 Western blot (Article IV)

For Western blotting mandibular molars from PN3 mice were microdissected in Dulbecco's PBS medium under a stereomicroscope, snap-frozen in liquid nitrogen and stored at -80^{o} C. Dental pulps from 10 to 22 mandibular first molars of the same developmental stage were extracted to obtain a sufficient amount of protein. Protein extracts were obtained after complete homogenization in 150µl of fresh RIPA buffer (50mM Tris-HCl pH 8, 150mM NaCl, 0.5% Doc, 1% NP40, 0.1% SDS) and mixture of protease inhibitors (phenylmethylsulfonyl fluorid, leupeptin, aprotinin and pepstatin). Samples were then centrifuged at 10 000 rpm for 15 min at 4°C. For each sample, the total soluble protein concentration was measured using Coomassie Plus Protein Assay Reagent Kit (1856201 Pierce, USA). Loading buffer (2% SDS, 10% Glycerol, 50 mM Tris-HCl pH 6.8, and 0.1% Bromophenol blue (non-reducing conditions)) was added to equally concentrated samples (80µg of protein extract) and samples were boiled for 5 min, vortexed and cooled in ice. 20µl of each sample was loaded into each well. Subsequently, the samples were electrophoresed in 8% SDS-PAGE gel. The proteins

were transferred onto nitrocellulose membrane (Bio-Rad, CA) utilizing mini Trans-Blot Electrophoretic transfer cell. Molecular weight marker (Bio-Rad) (Amersham Corp, Arlington Heights, IL) was run together with the samples. To avoid non-specific binding, membranes were blocked using 5% non-fat dried milk for 1 hr at room temperature. Anti-Sema4D, anti-Plexin-B1 and -B2 antibodies were used as primary antibodies and incubated overnight at 4°C and afterwards washed with PBS-Tween 3 x 10 min. The incubation time for horseradish peroxidase conjugated secondary antibodies was 1 hr at room temperature. All secondary antibodies were diluted in blocking buffer at a dilution of 1:10000. Signals were visualized with enhanced Super Signal Chemiluminescence Kit (34096 Pierce, USA) and membranes were analyzed using an Image Reader (Las-3000 version 2.0W).

3.7 In situ hybridization (Articles I, II, III and IV)

In situ hybridization was used to analyse the cellular localization of mRNAs of interest. It was performed according to the protocol described earlier (Luukko et al., 1996; Kettunen and Thesleff, 1998). In brief, different plasmids containing the subcloned cDNA fragments were linearized with different restriction enzymes and ^{35}S-UTP-labelled sense and antisense riboprobes were produced by using *in vitro* transcription using PS6, T3 or T7 RNA polymerases (Promega Corp., Madison, USA). Subsequently, the probes were precipitated using ethanol, air dried and dissolved in 1M dithiothreitol (Sigma-Aldrich, MO, USA) in hybridization buffer. The concentration of each riboprobe was diluted to 40-50 X 10^3 cpm/ul, being measured in a scintillation counter. The deparaffinized sections were treated with proteinase K (Promega Corp., Madison, USA). Sections were hybridized with riboprobe in a volume of 20-100µl at 52°C for 15-24 hours. Thereafter, the sections were incubated in 20mM dithiothreitol, 50% formamide and 2 x SSC at 65°C for 30 min. The dehydrated and air-dried sections were covered with NTB2 autogradiography emulsion (Eastman Kodak, New Haven, USA), Afterthree-to-four-week-exposure time, the autogradiography emulsion was developed in D-19 developer and fixed in Unifix (Eastman Kodak, New Haven, USA). They were counterstained with hematoxylin, and mounted with Depex (BDH, VWR international

Ltd, Dorset, UK). Control sections hybridized with sense probes showed no specific hybridization signal.

The used plasmids contained cDNA fragments of the following genes: Ngf, Gdnf, Ncam, TrkA, p75, Ret, Npn1, PlxA4, Npn2, TrkB and TrkC cDNAs (Luukko et al., 1996; Luukko et al., 1997b; Kettunen et al., 2005) (Luukko et al., 1997a) (Sijaona et al., 2012), Plexin-B1, Plexin-B2, Plexin-B3 (Perala et al., 2005), Sema3A (Loes et al., 2001), Sema4A, Sema4B, Sema4C, Sema4D, Sema4G and Sema4F (were used as templates to prepare riboprobes for *in situ* hybridization. The plasmids containing Sema4A, -4B (Skaliora et al., 1998), -4D (Worzfeld et al., 2004), -4G, and Sema4C, as well as Sema4F (Encinas et al., 1999) were generously provided by Drs. A.W. Puschel, and T. Kimura.

3.8 Quantification of nerve fibers (Article III)

The areas of nerve fibers in the pulp and periodontium at PN0 in the mandibular incisor were quantified from digital images of the immunostained sections taken using a 10X objective in a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Jena GmBH, Jena, Germany) equipped with a Spot insight digital camera (Diagnostic Instruments Inc, Sterling Heights, MI, USA). Three representative coronal sections from the anterior (incisal), middle and posterior (close to the cervical loop) regions of *Sema3A*$^{-/-}$ (n= 3) and *Sema3A*$^{+/+}$ (n= 3) incisors were blindly analyzed using Adobe Photoshop CS4 Extended program. In brief, the pixel width in micrometers was defined using Set Measurement Scale function of the program and scale bar (Leica Microsystems, Germany). Periodontal space and pulp regions were manually defined using the lasso tool (Adobe Photoshop CS4 Extended). Threshold levels of the images were then adjusted to select the nerve fibers and, when relevant, manual corrections were performed. The selected areas were measured using Record Measurements function. The areas of nerve fibers in the pulp and periodontium in μm^2 were exported to Microsoft Excel 2003. The data obtained were analyzed using SPSS software (IBM, USA) and presented as mean ± robust standard errors.

3.9 Computed tomography of whole heads (Article III)

Adult (18 months) *Sema3A*$^{+/+}$ (n= 3) and *Sema3A*$^{-/-}$ (n= 1) mice were euthanized using carbon dioxide, and the heads were stored at -80°C. Computed tomography scan of whole heads was performed with nanoScan small animal PET/CT scanner (Mediso Medical Imaging System, Budapest, Hungary). 720 projection images through 360° were taken using tube voltage of 70 kVp. The used exposure time was 450 ms per projection image and 1:1 binning was used. Images were reconstructed real-time at 20 μm^3 resolution using Nucline software (Mediso Medical Imaging System, Budapest, Hungary) and three dimensionally visualized with maximum intensity projection method using InterView Fusion software (Mediso Medical Imaging System, Budapest, Hungary). Enamel and dentine thicknesses were measured at the middle areas of the mandibular incisors from the coronal slices using InterView Fusion software.

3.10 Image processing

Images for *in situ* hybridization results were taken under dark- and bright-field microscopy, (Kettunen et al., 2005) using Spot insight digital camera (Diagnostic Instruments Inc, Sterling Heights, MI, USA) mounted on a Zeiss Axioskop 2 (Carl Zeiss Jena GmBH, Jena, Germany). Objectives with 5X and 10X magnification were used. Photomicrographs of the immunostained sections were taken using the same camera and microscope. For immunofluorescence, imaging was executed with confocal microscope (Leica SP5 AOBS) using 488 nm Argon ion and 561 nm DPSS lasers. Sequential scanning method was used to prevent crosstalk and bleed-through. Differential interference contrast image (DIC) of the same section was taken in a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Germany). Optical sections were volume-rendered with maximum intensity projection method using the Imaris software (Bitplane, Zurich, Switzerland). In order to animate immunofluorescence results Imaris software (Bitplane, Zurich, Switzerland) was used and images were additionally further

processed with Quicktime Pro software (Apple Inc, USA). Two-dimensional snapshots of volume rendered images were taken to make an image plate with Adobe Photoshop CS4 software (Adobe systems, San Jose, USA).

3.11 Methodological considerations

3.11.1 Animal model of study

The mouse (Mus musculus) has been used as the key animal model in genetic and developmental studies and is considered as the main model organism for performing developmental and genetic studies in mammals (Klein et al., 2013). This is because the mouse has a very similar anatomy, physiology and genetics compared to that of human beings. The published mouse genome sequence showed that over 95% of the mouse genome is similar to that of humans (Mouse Genome Sequencing Consortium, 2002 Nature). Like human mandibular molars, the mouse first mandibular molar has a multi-cusped crown and two roots. The mouse mandibular incisor has been used to analyse the function of stem cells during organ development. Consequently, mouse mandibular incisor and first molar tooth germs have been used as experimental models in this study. Mouse strains, namely BomTac:NMRI, the wild type (The Naval Medical Research Institute) albino outbred strain from Taconic Europe A7S, Denmark and Sema3A deficient mouse strains in C57BL/6 and CD1 background were used (Taniguchi et al., 1997).

Good arguments for choosing a mouse as a study model are short reproductive cycle length, relatively large litter size, small body size, easy manoeuvrability and uncomplicated maintenance of breeding colonies in the animal facility. Disadvantages, on the other hand, may include high maintenance and housing expenses, especially if various mouse colonies are housed in parallel in the vivarium. In addition, tasks relating to taking care of the colony such as ear marking, arranging breeding cages, checking

plugs, weaning, identifying and separating female and male pups as well as genotyping are time-consuming tasks.

3.11.2 Visualization of mRNAs in the tissues

Cellular expression of mRNAs of interest was examined by employing *in situ* hybridization technology. The technique may be utilized to assess localization and production of transcripts in time and space in paraffin or cryosections, as well as in tissue, organ and embryo whole mounts (Wilkinson et al., 1990). Oligoprobes or single-stranded cRNA probes are currently commonly used. The probes are labelled with radioactive isotopes such as ^{32}P, ^{35}S or ^{3}H. An alternative non-radioactive method is to label the probe with biotin and digoxigenin, and visualize the transcripts with alkaline phosphatase conjugated antibody. In addition, fluorescence *in situ* hybridization (FISH) is feasible at least in mouse whole embryos up to about mid gestation (Neufeld et al., 2013). ^{35}S-labeled riboprobes usually give low noise and a good signal. The disadvantage is that mRNA expression of only one gene can be analysed in each sample at one time and that tissue sections, not whole tissues, organs or embryos can be examined. The advantage of a non-radioactive method is that mRNA expression of at least two different genes can be examined concurrently in one specimen, and the method can be employed using whole tissues, organs or embryos. If the technique is employed on tissue sections better histology is obtained in paraffin sections compared to cryosections. Yet, use of nonradioactive probes on cryo-sections may be preferred in case of time limitation.

mRNAs in samples may be studied by some other methods such as RT-PCR (reverse transcription-polymerase chain reaction) (Wong and Medrano, 2005), microarray (Robert, 2010), Ribonuclease protection assay and Northern blot analysis (Reue, 1998). The most sensitive, accurate and rapid assay available for the detection of mRNAs is RT-PCR. On the other hand, reverse transcription quantitative-PCR requires careful and time-consuming planning in order to provide biologically meaningful and reliable

results (Derveaux et al., 2010). Because of high sensitivity it may give false positive results. This concerns also genotyping of mice by PCR. In addition, it does not provide information of the cellular localization of the transcripts in tissues or organs, which contain various cell types. With the high-throughput method, microarray, it is possible to analyse and compare the presence and levels of hundreds or even thousands of mRNAs in one sample whereas the key shortcoming is that it does not reveal cellular localization of transcripts. Commercial microarray chips are expensive and hybridization and analysis require specific devices. RNAase protection assay and Northern blot analysis were more frequently used earlier, especially before development of the RT-PCR technique. Both assays need relatively large amounts of starting material, which is a disadvantage compared to RT-PCR and microarray techniques. Northern blot allows determination of mRNA size and splicing variants (Streit et al., 2009). It is also sensitive, but Ribonuclease protection assay has been suggested to be at least 10-fold more sensitive than Northern blot.

In situ hybridization performed on sections was the most applicable method to investigate transcript expression in postnatal mouse teeth. In addition it was the only method available that could be used to visualize the molecules of interest, since antibodies against semaphorins, able to work on tooth sections do not exist. There are publications, which suggest that the assay is also suitable for mRNA quantification on sections (Vizi et al., 2001). Disadvantage was use of radioactive isotope, which has short life-time and is expensive, in labeling the probes. In addition, the assay is time-consuming and due to long exposure time (3-4 weeks) experiments could be executed on a relatively seldom basis.

3.11.3 Visualization of neurites

The first technique to be utilized to make neurons and neurites visible was a silver impregnation procedure discovered by Camillo Golgi (Torres-Fernández, 2006). This is still recognized as an excellent approach in order to visualize pathological morphology

of neurons such as degenerating neurons and their neuritis, in addition to normal ones (Tenkova and Goldberg, 2007). The advantage of the method is that it is less expensive, but on the other hand it is regarded as a demanding technique to handle (Tenkova and Goldberg, 2007).

Immunohistochemistry is a method of choice nowadays to visualize neurites in histological sections or whole mounts (tissue, organ or embryo), using antibodies against neurone and neurite specific proteins. Polyclonal rabbit anti-Peripherin antibody and monoclonal mouse anti-Neurofilament 200 antibody were used in this study to immunolocalize dental neurites. The antibodies were co-localized in the embryonic and early postnatal dental neurites, but anti-Neurofilament 200 gave a weaker positive immunoreaction. Neurofilament 200 is a cytoskeletal 200 kDa intermediate filament protein restricted to neurons and their processes (Herrmann and Aebi, 2000). Peripherin is a cytoskeletal 57 kDa class-III intermediate filament protein found especially in PNS but also in CNS neurones (Parysek and Goldman, 1988). There are also other neurone-specific antibodies that can be used. Beta-tubulin III (Tuj1) is a 50 kDa protein which belongs to the tubulin superfamily. Anti-Tuj1 antibody stains neurones and their processes both in CNS and PNS. It is not exclusively neurone specific since it also stains dendritic cells in skin (Lauria et al., 2004). Microtubule-associated protein Tau is localized in neurones, and especially in axons. Anti-Tau antibody has been applied to stain sensory dorsal root ganglion neurones (Georgieff et al., 1993). Sensory dental neurites in the dental pulp of the postnatal tooth germs have also been immunostained with 2H3 neurofilament antibody (Moe et al., 2008). Furthermore, antibodies against neuropeptides such as calcitonin gene related peptide (CGRP) and substance P (SP), have been employed to localize dental neurites in older postnatal and adult mature teeth (Kvinnsland and Heyeraas, 1992; Veerayutthwilai et al., 2006).

Anti-PGP9.5 (Protein gene product 9.5) antibody is a non-specific neuronal marker, which stains both sensory and sympathetic neurites. The antibody has been used to visualize neurites in the adult mouse and human teeth. A disadvantage is that it seems to produce unspecific staining in odontoblasts and outer dental epithelium (Fristad et al.,

1994; Ohshima et al., 2001; Moe et al.). Earlier it has been reported that the antibody appears not to stain thin developing immature sensory dental neurites during embryogenesis (Loes et al., 2002).

3.11.4 Presence of proteins in tissue samples

Immunoblotting (Western blotting), immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA) are commonly used assays to investigate proteins in tissue samples. These methods identify the antigen protein in the tissue sample on the basis of its interaction with a specific antibody. For immunoblotting, proteins are first isolated and then run and separated in detergent sodium dodecyl sulfate (SDS) containing buffer, which unfolds and negatively charges proteins. If separation of proteins is done under reducing conditions the loading buffer contains either beta-mercaptoethanol or dithiothereitol (DTT). It is a useful assay for the analysis of dimerization and oligomerization of proteins. Immunoblotting enables one also to study molecular weight, concentration, phosphorylation and posttranslational modifications of proteins. After blotting to a membrane, proteins of interest are nowadays commonly immunodetected with either direct or indirect methods using a horseradish peroxidase conjugated antibody, and immunoreactive bands are visualized with chemiluminescence in a specific analyser (Kurien and Scofield, 2006).

The advantage of immunohistochemistry on sections or whole mounts is that it is possible to visualize proteins in their native locations. This information can be used, for example, to predict functions of the proteins. Most primary antibodies work in immunoblotting, and there are more problems with antibodies in immunohistochemistry. Each antibody has to be tested in order to establish the optimal working conditions. Tissue specimens can be fixed in formaldehyde based or precipitating fixative such as ethanol, methanol or acetone. They can be embedded in paraffin or OCT (Optimal cutting temperature). Antigen retrieval, which may be executed with enzyme or microwave treatment, is commonly needed in formaldehyde

fixed tissues in order that the antibody may recognize the antigen. Monoclonal antibodies are very specific, since they recognize only one epitope on the antigen, and therefore provide less background, but also give a poorer signal. The advantage of using polyclonal antibodies is that they result in a strong staining reaction. They may, however, produce an intense background as well (Harlow and Lane, 1999).

In ELISA, a sample antigen, which is immobilized on a microtiter well plate, is analyzed with a specific enzyme-conjugated antibody. Alternatively the antibody could be immobilized. After adding a substrate a positive signal is detectable due to a color change. The method has been widely used as a diagnostic tool in clinical medicine (Lequin, 2005).

In addition, mass spectrometry provides a sensitive method to identify proteins. It is applied to the protein sample that has been run and separated in two following steps according to the charge and size of the proteins (O'Farrell, 1975). Detected amino acids are compared to protein databases using computer technology, which enables identification of known and unknown proteins in the sample. The potential is that an unknown protein can be investigated and, furthermore, a high number of proteins can be studied simultaneously. Using this technique there is no need for antibodies.

3.11.5 Analysis of protein functions during organogenesis

Functions of proteins can be assessed *in vitro* or *in vivo*. Typical *in vitro* assays are performed on cell, tissue, and organ cultures. They are more feasible from time and expense standpoints compared to *in vivo* assays. There are several model organisms, such as *Cenorhabditis elegans, Drosophila melanogaster, Xenopus laevis, Danio rerio, Gallus gallus domesticus, Mus musculus and Rattus norvegicus*, cells of which are used to investigate *in vivo* roles of proteins during development. Currently the most frequently used mammal in developmental studies is the mouse. In order to study developmental functions of proteins *in vivo*, transgenic mouse strains such as knock-out

and knock-in mice are used. The disadvantage with transgenic mice is that usually no more than one or two proteins can be investigated simultaneously. It is however in some cases possible to generate triple and quadruple knock-out mouse strains (Schluter et al., 2004). In this study, the *in vivo* roles of Sema3A in establishment of dental innervation during postnatal odontogenesis was addressed using Sema3A knock-out mouse strains in CD1 and C57BL/ background (Taniguchi et al., 1997). Our results show that background of the mice had no effect on the dental innervation phenotype. To our knowledge no *in vitro* experiment exists which could have replaced the analysis of dental innervation phenotype in Sema3A knock-out mice. In cases where transgenic mice may die very early during embryogenesis, *in vitro* experiments are very beneficial, since it is feasible to perform them at later stages of development. On the other hand, corroboration of *in vitro* experiments can be carried out with transgenic mouse technology. The drawback with the technology is that it is very expensive and generation of the mice requires cutting-edge expertise in complex cloning methods and mouse genetics.

4. RESULTS

4.1 mRNA expression of Sema3A, class 4 semaphorins and receptors in the developing tooth germ as well as detection of Sema4D protein and its receptors in the trigeminal ganglion

4.1.1 mRNA expression of Sema3A in the developing mouse incisor (Article I)

At embryonic day 11 (E11) and E12, Sema3A transcripts were observed in the epithelium and mesenchyme of the mandibular process. The mRNAs were detected in the oral epithelium and mesenchyme, including the presumptive areas of the incisor tooth germs, at E11. However, *Sema3A* was mostly absent from the middle core area of the mandibular process mesenchyme. At E12 some Sema3A mRNAs were seen in the presumptive dental mesenchyme and in the epithelial bud. A marked expression of *Sema3A* was also observed in the mesenchymal condensate of the developing Meckel's cartilage. At the bud stage (E13), Sema3A mRNAs showed a prominent hybridization

signal in the central core region of the dental epithelium. In addition, some transcripts appeared in the condensed dental mesenchyme (presumptive dental pulp mesenchyme) and in the surrounding jaw mesenchyme corresponding to the presumptive area of the developing alveolar bone. At E14 and E16, *Sema3A* appeared in the outer dental epithelium extending to the cervical loop, as well as in the developing alveolar bone. Transcripts were also seen in the epithelial stellate reticulum at E16 but no specific expression was observed in the mesenchymal dental papilla or follicle. Postnatally, at PN0, PN4 and PN5, expression of Sema3A mRNAs continued in the outer dental epithelium and in the cervical loop, both on the labial and lingual sides. The developing alveolar bone continued to exhibit *Sema3A* expression. Of note, no specific expression *Sema3A* was observed in the mesenchymal dental pulp including the area of the apical foramen. The developing dental follicle target area was found to be devoid of hybridization signal throughout the study of the embryonic and postnatal stages.

4.1.2 mRNA expression of class 4 semaphorins and plexin receptors in the molar anlage (Article IV)

4.1.2.1 mRNA expression of class 4 semaphorins in the early postnatal mouse mandibular molars

Sema4A mRNAs were predominantly expressed in the dental epithelial tissue components of the first molar tooth germ. At PN0 and PN1, Sema4A mRNAs appeared in the inner and outer dental epithelia, stellate reticulum, stratum intermedium and cervical loop whereas preameloblasts were devoid of transcripts. Sema4A transcripts were also observed in the mesenchymal cells of the middle part of the dental papilla. At PN2, a weaker expression of *Sema4A* was detected in the dental epithelial cells except the cervical loop where a marked expression continued. At PN5 and PN7 enamel and dentin were visible in the tooth germs. HERS epithelium (Hertwig's epithelial root sheath) displayed an apparent *Sema4A* expression, but in other dental epithelial and

mesenchymal cells expression was no more clearly detectable. A positive *Sema4A* expression was also detected in the oral epithelium during the studied stages.

Sema4C mRNAs showed no specific hybridization signal during early postnatal ages but some transcripts were seen in the post-secretory ameloblasts at PN7.

Sema4D mRNAs showed a high level of expression in the preodontoblasts at PN0 and PN1, One day later expression was confirmed in the odontoblasts. The expression of *Sema4D* was also apparent in the radicular odontoblasts at PN5 and PN7. A much weaker *Sema4D* expression was seen in the stratum intermedium, stellate reticulum and outer enamel epithelium at PN0 and PN2, and later, at PN5 and PN7, a notable expression of *Sema4D* was seen in the ameloblasts. The presence of the protein product of *Sema4D* was confirmed in postnatal molar tooth germ (PN3) using Western blot analysis. Sema4D transcripts also appeared in the large and small diameter cells of the developing bone, suggesting putative expression in the osteoclasts and osteoblasts, respectively, as well as in the oral epithelium at all stages studied.

Sema4B mRNAs showed ubiquitous hybridization signals in the molar tooth germs as compared to control sections during the studied stages. No specific hybridization signal Sema4F and Sema4G mRNAs was observed.

4.1.2.2 Expression of PlexinB1-B3 receptors in the early postnatal molar tooth germ

Expression of PlexinB1 and PlexinB2 mRNAs was mostly restricted to the dental epithelium of the molar tooth germ throughout the studied stages. At PN0-PN2, faint *Plexin-B1* and *PlexinB2* expression was seen in the enamel organ including the inner and outer dental epithelia, preameloblasts, cervical loop, stratum intermedium and stellate reticulum. Later at PN5 and PN7 expression of both mRNAs was observed in the enamel-secreting ameloblasts. In addition, the Hertwig's epithelial root sheath displayed *PlexinB1* expression. A few PlexinB1 and -B2 transcripts also appeared in

the dental papilla and pulp during PN0-PN2. The developing alveolar bone also expressed *PlexinB1* and *-B2*. The presence of PlexinB2 protein was identified in molar tooth germ (PN3) using Western blot. No specific hybridization signal of *PlexinB3* was observed in the developing molar or adjacent tissues.

4.2 mRNA expression of neuroregulatory molecules and their receptors in Sema3A-deficient incisors, molars and trigeminal ganglia (Articles II and III)

The cellular mRNA expression patterns of selected key, putative tooth innervation regulating molecules namely Ngf, Gdnf and Ncam were compared in Sema3A$^{+/+}$ and Sema3A$^{-/-}$ incisors (PN5) and molars (PN0, PN1, PN3 and PN5) using sectional *in situ* hybridization. Transcripts of all studied mRNAs were observed in incisor and molar tooth germs of both genotypes, and no apparent changes in their expression patterns were observed. *Ngf* expression was observed in the subodontoblastic area of the molar tooth germ at PN1 and in the later stages. Gdnf transcripts were also seen in the area, whereas *Ncam* showed a notable, broad expression in the dental pulp including the odontoblasts as well as in the dental follicle. Similarly, no apparent differences in the mRNA expression of the studied molecules between the postnatal Sem3A$^{-/-}$ and Sema3A$^{+/+}$ incisors were detected.

Expression of signaling receptors of Ngf, Gdnf and Semaphorin families were investigated in PN3 Sema3A$^{+/+}$ and Sema3A$^{-/-}$ trigeminal ganglia. All TrkA, TrkB, TrkC, p75 (LANR), Ret, Npn1, Npn2 and PlxnA4 receptor mRNAs were found in the ganglia in both genotypes. No apparent differences in their cellular expression patterns between the genotypes were observed.

4.3. Localization of neurites in the Sema3A-deficient incisor and molar tooth germs (Articles II and III)

The distribution of neurites was investigated in the incisor and molar tooth germs using immunohistochemistry on sections. At E11.5, nerves were not seen in the mesenchyme close to the presumptive incisor epithelium area in the Sema3A$^{-/-}$ or wild-type embryos but they were widely distributed in the Sema3A$^{-/-}$ mandibular process mesenchyme. One day later at the bud stage, neurites were still not detected in the vicinity of the developing *Sema3A*$^{+/+}$ tooth germ. In contrast, some ectopic nerve fibers were present in the presumptive Sema3A$^{-/-}$ incisor dental mesenchyme. Nerve fibers were not seen to have reached the dental epithelium.

At the cap and bell stage (E13.5 and E16.5, respectively), nerve fibers were located in the dental follicle target field area in the both Sema3A$^{-/-}$ or wild-type incisors. In the Sema3A$^{-/-}$ tooth germ many nerve fibers were abnormally localized in the dental papilla mesenchyme. Moreover, a higher number of nerve arborizations was evident in the Sema3A$^{-/-}$ incisors than in the corresponding wild-type ones. In *Sema3A*$^{-/-}$ embryos, ectopic nerve fibers were also observed in the mesenchymal areas of the developing mandibular and alveolar bone.

In the postnatal PN0 and PN2 Sema3A$^{-/-}$ incisors, nerves were present in the middle part of the dental pulp, whereas they were absent from the wild-type pulps. There were also more nerve fibers in the developing periodontium of Sema3A$^{-/-}$ incisors than in the wild-type ones. Whereas in the wild-type incisors only a few nerves, if any, appeared in the periodontium of the labial side of the tooth germs, they were noted in the *Sema3A*$^{-/-}$ periodontium in both labial and lingual sides. Measurement of the areas occupied by nerve fibers showed that the values for the *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ *PN0 incisor* pulps were 0±0 µm2 and 54.64±28.60 µm2, respectively. For the *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ incisor periodontium the following values were recorded: 5240.98±3467.69 µm^2 and 17412.75±6969.17 µm^2, respectively.

Nerve fibers were seen inside the dental pulp in the wild-type incisor for the first time at

PN3, and they were similarly located in the middle area of the pulp in both Sema3A$^{-/-}$ and Sema3A$^{+/-}$ genotypes. No subodontoblastic plexus formation or presence of nerve fibers in the developing dentin was seen. On the other hand, a higher number of nerve fibers was apparent in the Sema3A$^{-/-}$ periodontal space, in particular in the labial side of the tooth germ as compared to wild type teeth. Nerve fibers were not seen in the dental epithelium or mineralized alveolar bone. At PN5 and PN7, although the number of nerves in the Sema3A$^{-/-}$ dental pulp and periodontal space appeared to be higher, the pattern and localization of nerve fibers in the dental pulp was similar in both genotypes. In a similar fashion adult *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ pulp and the periodontal space exhibit similar innervation. Moreover, no abnormalities in Ruffini endings (Maeda et al. 1999) in Sema3A$^{-/-}$ incisors were apparent as compared to *Sema3A*$^{+/+}$ incisors.

In the *Sema3A*$^{+/+}$ first molar tooth germ, nerve fibers were not observed in the dental pulp at postnatal day 0 or 2. In contrast, they were evident in the Sema3A$^{-/-}$ and Sema3A$^{+/-}$ pulps already at PN0. Analysis of serial sections revealed that nerve fibers had entered the molar pulp from the mesial and distal ends of the tooth germ and some of them had even reached the presumptive pulp-dentin border area. Moreover, some axons were evident within the preodontoblast layer and close to the basement membrane next to the inner enamel epithelium. Moreover, some nerve fibers in the pulp were not heading to the coronal areas but rather in random directions.

Nerve fibers were found in the molar pulp of the wild-type mice for the first time at PN3. Serial sections revealed that they had penetrated the pulp, like in the Sema3A$^{-/-}$ molar, through the mesial and distal ends of the tooth germ. At this stage, the number of apparently defasciculated nerve fibers inside the dental pulp was found to be increased in Sema3A$^{+/-}$ and in particular Sema3A$^{-/-}$ molars. The pattern of nerves in the pulp looked disorganized, and many apparently defasciculated nerves were seen at the base of the dental pulp and at the pulp–dentin border area. In contrast, in the wild-type molar, the first nerve fibers had appeared in the border area only by PN5.

At PN5 and 7 the number of nerve fibers increased in the dental pulp in all genotypes. Nerves appeared thinner and defasciculated in Sema3A$^{-/-}$ pulp as compared to that of wild-type molars. In PN7 Sema3A$^{+/+}$ molars some nerve arborizations were seen at the pulp-dentin border area. In contrast, arborizations of nerve fibers were notable at the pulp-dentin border area in Sema3A$^{+/-}$ and especially in Sema3A$^{-/-}$ molars. Moreover, a wide, enlarged area of many apparently arborized nerves extending from the subodontoblastic area towards the deeper, core part of the dental pulp was evident in Sema3A$^{+/-}$ and in particular in the Sema3A$^{-/-}$ molar. In Sema3A $^{-/-}$ mice, nerves in this area exhibited an abnormal, fragmented appearance as compared to nerve fibers in the wild-type dental pulp, as also observed using thick confocal immunofluorescence microscopy.

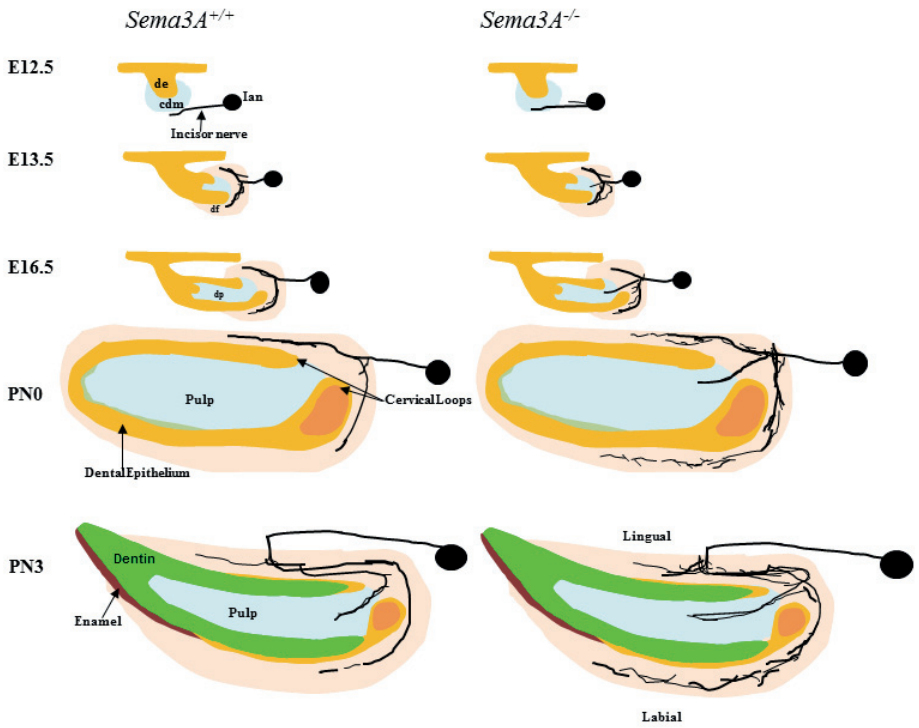

Figure 10. Schematic illustration of the distribution of nerve fibers (black) in embryonic and early postnatal Sema3A$^{+/+}$ and Sema3A$^{-/-}$ mandibular incisors. Abbreviations: cdm, condensed dental mesenchyme; de, dental epithelium; df, dental follicle; dp, dental papilla; ian, inferior alveolar nerve. Modified from (Wang et al., 2004).

4.4 Analysis of Sema3A-deficient developing and adult tooth (Articles II and III)

Sections of immuno- and haematoxylin-eosin stained embryonic and postnatal Sema3A-deficient and wild-type incisor and postnatal molar tooth germs were studied to address histomorphology of the tooth germs. In addition, computed tomography and visual observations were applied to study gross anatomy of the teeth as well as enamel and dentin. No apparent defects were found in morphology or histology of the Sema3A-deficient teeth or neighboring alveolar bone when compared to the wild-type.

4.5 Localization of Sema4D as well as PlexinB1 and -B2 receptors in postnatal trigeminal ganglion (Article IV)

Cellular localization of Sema4D and PlexinB1 and PlexinB2 receptors was studied in the trigeminal ganglion at PN2, PN5 and PN7 using sectional immunohistochemistry. Positive Sema4D, PlexinB1 and -B2 immunoreactivity was observed in the trigeminal neurons at all stages studied, namely at PN2, PN5 and PN7.

5. DISCUSSION

5.1. Sema3A signaling in developing incisor tooth germ innervation

The mouse incisor is a specific type of tooth that differs from the molar in different aspects. The incisor has a single apical foramen, it erupts continuously throughout life and production of enamel take place exclusively only in the labial side (Amar et al., 1986; Harada et al., 1999). Because developmental regulation of odontogenesis and discrete tooth phenotypes are dependent on distinct, differential molecular regulation (Salazar-Ciudad and Jernvall, 2002) (Thesleff et al., 2007), developing incisor tooth

germ was studied to investigate functions of Sema3A during incisor development and in odontogenesis in general.

Sema3A is an essential regulator of the trigeminal peripheral innervation during embryonic development (Taniguchi et al., 1997) (Kettunen et al., 2005). Sectional *in situ* hybridization analysis revealed developmentally controlled Sema3A expression domains during embryonic and postnatal development of the incisor. Sema3A mRNAs were found in the rostral part of the developing lower jaw including the presumptive area of the incisor tooth germ, suggesting that Sema3A may be involved in the regulation of early stages of development of incisor nerve supply. Indeed, it was found that nerve fibers were ectopically present in the presumptive incisive mesenchyme area of *Sema3A*$^{-/-}$ embryos at E12.5. In contrast, in the wild-type incisor the first axons were observed in the tooth germ area at E13.5. Thus, Sema3A appears to act as a locally produced, secreted chemorepellent cue to guide growing presumptive incisor dental neurites. As shown earlier during embryonic development (Tran et al., 2007) (Fujisawa, 2003) and also for the embryonic molar tooth germ (Kettunen et al., 2005), it is likely that Sema3A, acting through the developmentally changing exclusion areas, regulates early incisor tooth germ innervation by guiding nerve fiber growth to specific pathways, controls patterning of the neurites and thereby also determines the timing of the initial nerve fiber encounter to the incisor tooth germ. These results, and proposed functions, are in line with earlier data regarding functions of Sema3A in the embryonic mouse mandibular first molar tooth germ (Kettunen et al., 2005).

It was also noted that the number of ectopic nerve fibers observed in the early developing Sema3A$^{-/-}$ incisors appeared to be lower when compared to that of Sema3A$^{-/-}$ molars (Kettunen et al., 2005). This suggests that there exist some developmental differences between the tooth types regarding molecular regulation, such as in cellular expression patterns of neuroregulatory molecules. The fact that epithelial-mesenchyme interactions control molar innervation (Kettunen et al., 2005) (Kettunen et al., 2007) raises the possibility that local mechanics are also involved in regulation of incisor

innervation and thereby tooth type specific innervation. Furthermore, because the dental epithelium governs tooth formation during early stages (Mina and Kollar, 1987) (Lumsden, 1988) (Kollar and Baird, 1970), regulation of Sema3A or other putative incisor innervation regulating molecules may take place by dental epithelium expressed signaling molecules such as Wnt, Bmp and Fgf family members, which have been shown to mediate odontogenic epithelial-mesenchymal interactions and regulate tooth innervation by controlling mesenchymal Sema3A (Vainio et al., 1993) (Tucker et al., 1998) (Kettunen et al., 2005) (Kettunen et al., 2007). It is also tempting to propose that local signaling within the tooth target also coordinates advancing histomorphogenesis of the incisor with its innervation. This hypothesis is supported by data from the analysis of Sema3A$^{-/-}$ and Fgfr2b$^{-/-}$ molars. In particular, in Fgfr2b$^{-/-}$ molar morphogenesis stops at the degenerated cap stage (Kettunen et al., 2007) and there are defects in both Sema3A expression domains and axon patterning demonstrating that Fgfr2b mediated signaling controls both tooth morphogenesis and tooth innervation. Tgfß1, which regulates *Sema3A* expression and dental cell proliferation (Kettunen et al., 2007), is downregulated from the Fgfr2b$^{-/-}$ molar. Moreover, *Tgfß1* is regulated by epithelial-mesenchymal interactions (Vaahtokari et al., 1991) and epithelial *Fgf4*, which is essential for tooth morphogenesis (Kratochwil et al., 2002) and is not expressed in the Fgfr2b$^{-/-}$ molar (Kettunen et al., 2007).

5.1.2 Sema3A signaling in incisor pulp innervation

It has been shown earlier using three-dimensional (3D) analysis that nerve fibers enter the pulp of the two-rooted mouse mandibular molar through the presumptive sites of the secondary apical foramina in the mesial and distal ends where the roots will develop (Luukko et al., 2008). *Sema3A* shows specific expression at the base of the pulp mesenchyme whereas transcripts are not present in the presumptive area of the secondary apical foramina before and during the early stages of nerve fiber ingrowth (Kettunen et al., 2005). Based on these findings and the reported, developmentally changing mRNA expression domains of Sema3A that correlate with nerve growth, as

well as its functions during embryonic stages of tooth innervation (Kettunen et al., 2005) (Kettunen et al., 2007), it was hypothesized that Sema3A signaling may serve a role in the innervation of incisor pulp. Analysis of the localization of nerve fibers revealed that nerve fibers were ectopically present in the condensed *Sema3A*$^{-/-}$ incisor dental mesenchyme, dental papilla and pulp during the embryonic and early postnatal tooth germs. In contrast, the first nerves were initially observed in the dental pulp of the *Sema3*$^{+/+}$ incisors postnatally. This observed "waiting period" is in line with the timing of the innervation of the molar tooth germ (Tsuzuki and Kitamura, 1991) (Mohamed and Atkinson, 1983) (Moe et al., 2008) (Luukko et al., 2008) and for instance function of Sema3A in the innervation of the tongue epithelium innervation (Dillon et al., 2004). Thus, besides regulating the timing and patterning of early incisor tooth germ innervation, Sema3A controls the timing of dental pulp innervation. Because Sema3A mRNAs were not found in the incisor dental papilla or pulp, Sema3A is apparently involved in regulation of this process by preventing premature innervation of the dental mesenchyme, which later forms the dental pulp.

Sema3A$^{-/-}$ mouse embryos have been reported to exhibit a marked defasciculation and arborization of various peripheral nerves, including branches of the trigeminal nerve (Taniguchi et al., 1997) (Haupt et al., 2010). In addition, an increased number of ectopically located nerve fibers has been reported in the developing mandibular molar suggesting that Sema3A might regulate processes such as dental nerve fiber fasciculation, arborization and patterning during development of incisor pulp nerve supply (Kettunen et al., 2005; Kettunen et al., 2007). However, no apparent differences were found between the distribution of neurites within the dental pulp of Sema3A$^{-/-}$ incisors as compared to Sema3$^{+/+}$ ones. A typical feature of innervation of the molar tooth is the formation of the subodontoblastic nerve plexus and presence of nerve fibers within the predentin and dentin (Byers and Narhi, 1999) (Hildebrand et al., 1995), whereas these are not present in the mouse incisor. Similarly, no formation of subodontoblastic plexus or innervation of dentin was found in Sema3A$^{-/-}$ or control Sema3A$^{+/+}$ incisors. Because *Sema3A* is additionally not expressed in the incisor dental papilla or pulp, it is concluded that Sema3A does not seem to serve any role in

processes such as in the patterning or fasciculation of the incisor pulp nerves. However, it can be speculated that the possible, putatively minor, defects in Sema3A$^{-/-}$ pulp innervation, suggested by the presence of ectopic nerve fibers in the dental papilla, may have become corrected or are masked, or were not detectable by the methods used.

The dental follicle mesenchyme is located between the epithelial enamel organ and dental papilla/pulp and developing alveolar bone and gives rise to the periodontium attaching the root(s) into alveolar bone. Sema3A mRNAs were markedly expressed in the developing alveolar bone and outer dental epithelium and a higher number of nerves were noted in Sema3A$^{-/-}$ follicle mesenchyme of the developing incisor as compared to Sema3$^{+/+}$ one. The nerves located in the Sema3A$^{-/-}$ dental follicle showed apparent defasciculation. Of note, nerve fibers were also ectopically present in the developing alveolar bone but not observed in the dental epithelium as reported earlier in the developing Sema3A-deficient molar tooth. Later, the number of nerve fibers remained high in the dental follicle/developing periodontium of Sema3A-deficient incisors, in particular in the labial side of the tooth germ. Thus, Sema3A is proposed to act locally, as an incisor tooth germ-produced chemorepellent that establishes dynamic exclusion areas and consequently regulates nerve fiber navigation and pathfinding into the future periodontal space by a surround repulsion mechanism, as also proposed during early molar innervation (Kettunen et al., 2005). Sema3A apparently also controls nerve fasciculation and arborization during innervation of the incisor dental follicle and the periodontium.

It has been reported earlier that even though defects in the innervation of Sema3A-deficient mice appeared to become mostly corrected during late embryonic stages (Ben-Zvi et al., 2013) (White and Behar, 2000), some abnormalities persist to postnatal stages (Haupt et al., 2010). In the present study, it was observed that abnormalities in the dental follicle and periodontal innervation of developing Sema3A$^{-/-}$ incisors become gradually unnoticeable by adulthood, and for instance Ruffini endings appeared to be

similar in both Sema3A$^{+/+}$ and Sema3A$^{-/-}$ incisors. In addition, whereas numerous ectopic nerve fibers were observed in the embryonic Sema3A$^{-/-}$ alveolar bone no such defects appeared in the adult Sema3A$^{-/-}$ incisor alveolar bone. Thus, it seems that the observed disturbances in the early incisor and alveolar bone innervation process become gradually, if at all, corrected, most likely by the influence, apparently changing, of other innervation regulating molecules. This may possibly involve mechanisms involved in the regulation of the plasticity of the tooth innervation (see for instance (Fried et al., 2000) (Luukko et al., 2005a) (Luukko et al., 2008) (Fried et al., 2007) (Nosrat et al., 1998) (Nosrat et al., 1997) (Byers, 1994).

Earlier results, mostly obtained from the analysis of the developing molars, have provided data showing that signaling molecules of different families serve important roles in regulation of tooth nerve supply (see for instance (Mitsiadis and Luukko, 1995). (Fried et al., 2000) (Kettunen et al., 2005) (Luukko et al., 2008) (Fried et al., 2007) (Nosrat et al., 1998) (Nosrat et al., 1997). Many of the key and putative important neuroregulatory molecules are expressed in the tooth target and regulate critical aspects of tooth innervation (see for instance (Mitsiadis and Luukko, 1995) (Fried et al., 2000) (Luukko et al., 2005a) (Luukko et al., 2008) (Fried et al., 2007) (Nosrat et al., 1998) (Nosrat et al., 1997). In this study, no apparent alteration in the expression of Ngf, Gdnf and Ncam mRNAs in Sema3A$^{-/-}$ incisor tooth germ and the expression of selected receptors trigeminal ganglia was observed using sectional *in situ* hybridization analysis. This suggests that the molecules studied here, in addition to other molecules belonging to different families, may be involved, as proposed earlier for the embryonic molar (Kettunen et al., 2005) and, in the present study, for the postnatal mouse molar (see below), in correction of the observed early innervation defects in the incisor tooth germ. The present finding that Sema3A signaling regulates key aspects of early incisor tooth innervation such as axon navigation and patterning supports the hypothesis that tooth innervation is controlled by redundant and apparently independent signaling of neuroregulatory molecules of various families (Fried et al., 2000) (Luukko et al., 2005a) (Luukko et al., 2008).

5.2 Sema3A controls the timing and patterning of molar pulp innervation

The dental pulp is an important and densely innervated peripheral target area for the dental nerves. Earlier, *Sema3A* was demonstrated in the middle area of the base of the pulp and around the secondary apical foramina in the molar tooth germ prior to and during nerve fiber penetration into the pulp (Loes et al., 2001; Kettunen et al., 2005). Investigations using postnatal Sema3A-deficient mice revealed that nerve fibers were prematurely present in *Sema3A*$^{-/-}$ and *Sema3A*$^{+/-}$ mandibular first molar already at the newborn stage before the normal initiation of neurite ingrowth into dental pulp in *Sema3A*$^{+/+}$ molars observed at around PN3. Thus, Sema3A regulates the timing of innervation of the molar pulp. Similarly, because neurites were located within the layer of preodontoblasts already at the newborn stage, Sema3A also controls the timing of innervation of the pulp-dentin border area. It is possible that some of the nerve fibers present in the newborn dental pulp were those that had entered the dental papilla and survived there during embryonic stages (Kettunen et al., 2005). Indeed, the prenatal molar tooth has been shown to express members of the neurotrophic factor families (Nosrat et al., 1997) (Nosrat et al., 1998) (Luukko et al., 1997b) (Luukko et al., 1998) (Luukko et al., 1997a) (Mitsiadis and Luukko, 1995) and are therefore likely candidates to support them. The observed defects in timing and patterning of pulpal innervation in C57BL/6 and CD1 mouse strains were similar suggesting that the phenotypes are not strain-dependent. Moreover, the finding that the innervation defects in early postnatal Sema3A$^{-/-}$ molars were more severe as compared to Sema3A$^{+/-}$ ones, proposes that Sema3A is haploinsufficient.

The localization of Sema3A mRNAs in the base of the postnatal pulp before the appearance of the secondary apical foramina suggested that Sema3A might serve an important function in regulation of the sites of the nerve fiber penetration into molar dental pulp (Kettunen et al., 2005) (Luukko et al., 2008). However, no differences in the distribution of penetrating neurites into dental pulp were observed between Sema3A$^{-/-}$

and Sema3A$^{+/+}$ molars. Nerve fibers were found to enter into the dental pulp specifically through the mesial and distal ends. Based on this finding, Sema3A is assumed not to serve a critical role in patterning of neurite ingrowth into the molar pulp through the presumptive sites of mesial and distal roots. The dental pulp expresses mRNAs of different neuroregulatory molecules, in particular members of different semaphorins, many of which are potent axon repellents, and therefore may act as chemorepellents for the dental trigeminal axons (Lillesaar and Fried, 2004). In support of this hypothesis, signaling receptors for semaphorins such as PlexinA3 and -A4, Npn1 and -2 are expressed in the postnatal trigeminal ganglion and mice deficient for Npn1 and -2 as well as PlexinA3 and PlexinA3/A4 appear all to exhibit abnormalities in peripheral trigeminal nerve projections (this study, (Sijaona et al., 2012) (Cheng et al., 2001) (Giger et al., 2000) (Sahay et al., 2003) (Yaron et al., 2005) (Kitsukawa et al., 1997) (Chen et al., 2000). Taken together the data suggest that the dental pulp expressed other semaphorins, and/or perhaps other neuroregulatory molecules may have compensated for the absence of Sema3A in the transgenic mice suggesting that the functions of Sema3A might be redundant.

The fact that Sema3A mRNAs are absent from presumptive sites of the mesial and distal roots (secondary apical foramina) also suggests that Sema3A signaling does not appear to determine the normal, postnatal timing of dental axon ingrowth into molar pulp (Kettunen et al., 2005) (Luukko et al., 2008) and that, instead of Sema3A, Ngf, based on its key functions in tooth innervation, might serve a critical role in this process. Ngf signaling is essential for dental pulp innervation (Matsuo et al., 2001). Ngf is a survival and differentiation factor, wchich triggers trigeminal neurite outgrowth and guides sensory growth cones towards Ngf gradient (Luukko et al., 1997a) (Nosrat et al., 1997; Lillesaar et al., 1999) (Tessier-Lavigne and Goodman, 1996) (Marsick et al., 2010) (Patel et al., 2000). Interestingly, in this study it was found that the induction of *Ngf* in the mouse mandibular first molar pulp took place prior to the initial nerve fiber ingrowth into dental pulp, and the level of expression increased during subsequent development. A similar finding has been reported in the developing rat molar (Nosrat et

al., 1998) (Luukko et al., 1997a). Interestingly, Ngf has been shown to be involved in the collapse response of sensory axon growth cones to Sema3A (Dontchev and Letourneau, 2002) (Dontchev and Letourneau, 2003). Thus, besides direct effects on nerve fibers, Ngf might also control tooth innervation by making dental nerve fibers less sensitive to the repellent influence of Sema3A (Dontchev and Letourneau, 2002) (Dontchev and Letourneau, 2003).

Dental pulp expresses many molecules, which are likely to have an influence on dental axon growth (Lillesaar and Fried, 2004) (Nosrat et al., 1998) (Lillesaar et al., 2003) (Lillesaar and Fried, 2004) (Luukko et al., 1997a) (Luukko et al., 1997b) (Luukko et al., 1998) (Sijaona et al., 2012). Of the putative molecules, besides Ngf, Gdnf, Bdnf, Ncam and laminin may have a positive effect on axon growth and thus be involved in dental pulp innervation for instance in the regulation of timing of nerve ingrowth (this study, (Lillesaar et al., 1999) (Fried et al., 2000) (Lillesaar et al., 1999). It is also likely that in the absence of repellent Sema3A signaling, Ngf and Gdnf, possibly by a synergistic mode of action, may contribute differentially to the appearance of the specific innervation abnormalities in Sema3A$^{-/-}$ teeth (Madduri et al., 2009). In embryonic chicken DRG explants, Gdnf promoted more axonal elongation whereas Ngf induced extensive branching of axons (Madduri et al., 2009). These and the earlier findings for instance that many molecular families such as morphogens and canonical axon guidance molecules (Charron and Tessier-Lavigne, 2005) (Giger et al., 2010) are critical for development of the nervous system providing further support for the hypothesis that tooth innervation is regulated via coordinated action of many different signal families (Fried et al., 2000) (Luukko et al., 2005a).

5.2.1 Sema3A controls fasciculation of the dental pulp neurites

It was also found that incisor and mental nerves showed obvious defasciculation in both *Sema3A*$^{-/-}$ and *Sema3A*$^{+/-}$ mice indicating that Sema3A signaling controls fasciculation of nerves supplying teeth. That pulpal nerves of Sema3A-deficient molars undergo

defasciculation postnatally and mRNAs for Sema3A signaling receptor PlexinA4 and co-receptor Npn1 are expressed in the PN5 trigeminal ganglion neurons (Sijaona et al., 2012) suggests that Sema3A regulates the patterning of the dental pulp nerves through Npn1-PlexinA4 receptor complex by influencing on their fasciculation. However, in one study postnatal dental neurites appeared to show no or very little Npn1 immunoreactivity (Lillesaar and Fried, 2004). Thus, as shown for the certain growth factors such as Fgfs during the formation of the tooth crown (Jernvall and Thesleff, 2000), this finding indicates that Sema3A signaling is iteratively used at different, key embryonic and postnatal stages of tooth innervation (Kettunen et al., 2005) (Kettunen et al., 2007).

Because *Sema3A* is absent from the middle core part of the dental pulp but is expressed around the presumptive areas of the secondary apical foramina in the postnatal molar (Kettunen et al., 2005) it is proposed that Sema3A, which is a diffusible molecule (Messersmith et al., 1995)**,** influences on dental pulp nerve fasciculation as they penetrate into the dental pulp.

5.2.2 Premature formation of nerve plexus at the coronal pulp-dentin border area in the Sema3A-deficient molar

The pulp-dentin border target area is a highly innervated target field in the dental pulp (Byers, 1984) (Fried et al., 2007) (Luukko et al., 2008) (Magloire et al., 2010) and an extensive arborization of axons is present in the subodontoblastic nerve plexus of Raschkow (Hildebrand et al., 1995). *Sema3A*$^{-/-}$ molar pulps showed an apparent, premature initial formation of nerve plexus in the pulp dentin border area at PN2, and during later postnatal development the nerve plexus appeared atypical and had enlarged towards the deeper, core area of the dental pulp as compared to *Sema3A*$^{+/+}$ molars. Even though Sema3A exerts inhibitory activity on axon branching (Gibson and Ma, 2011) and its mRNAs are expressed in the preodontoblasts present in the pulp-dentin border area from the embryonic stages (Kettunen et al., 2005) Loes, 2001 #265}, it is

downregulated in the odontoblasts and not detected in the most advanced coronal areas before the arrival of the first ingrowing nerve fibers in the wild-type molars (Kettunen et al., 2005). It is likely, therefore, that the observed abnormal branching of nerves at the *Sema3A*$^{-/-}$ pulp-dentin border area is mostly not dependent on Sema3A, but apparently due to influence of other neuroregulatory molecules reported in the target area, such as Ngf, Bdnf and Gdnf neurotrophic factors as well as semaphorins such as Sema7A and Sema4D (Kvinnsland et al., 2004) (Luukko et al., 1997b) (Mitsiadis et al., 1993) (Byers et al., 1992) (Nosrat et al., 1998) (Nosrat et al., 1996) (Nosrat et al., 2004) (Nosrat et al., 1997) (Lillesaar and Fried, 2004), this study) (Maurin et al., 2005) (Fried et al., 2000). In this study, *Ngf* and *Gdnf* expression in the subodontoblastic area in both *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ molars was found not to be altered and no apparent changes in the cellular expression of receptors (*TrkA, p75 and Ret*) were observed in the postnatal *Sema3A*$^{-/-}$ trigeminal ganglia. Ngf and Gdnf, though at lower levels, promote axonal branching of DRG sensory neurons. In addition, Ngf and Gdnf trigger neurite outgrowth in the postnatal rat trigeminal ganglion *in vitro* (Lillesaar et al., 1999). Furthermore, application of Ngf and Gdnf together elicit more intense neurite outgrowth from the trigeminal ganglia (Lillesaar et al., 1999) as also found in chicken DRG explants (Madduri et al., 2009). Thus, it is possible that the apparent increased nerve arborization and branching in the *Sema3A*$^{-/-}$ and *Sema3A*$^{+/-}$ pulp-dentin border target area may be due to a longer exposure of the neurites to the target field expressed neuroregulatory molecules, which may exert synergic effects. The data also show that the observed disturbances of the dental pulp innervation in Sema3A-deficient molars were not corrected by PN7, but instead they appeared to become more severe. Further studies regarding molar innervation in later postnatal and adult Sema3A-deficient mice are needed, especially detailed investigations of the functions, mode of actions and interactions of Sema3A signaling during tooth innervation.

5.2.3 Class 4 semaphorin signaling may regulate dental pulp innervation

Sema4A mRNAs were detected in the middle, core part of the early postnatal molar

pulp before the ingrowth of first neurites at around PN4. Moreover, immunoreaction of Sema4A signaling receptors PlexinB1 and -B2 was observed in the trigeminal ganglion neurons before PN3. Because membrane-bound Sema4A is able to induce growth cone collapse through Rho-kinase, as demonstrated for hippocampal neurons (Yukawa et al., 2005), it is tempting to speculate that Sema4A produced in the dental pulp could function as a chemo-repulsive factor on dental neurites and regulate pulp innervation. On the other hand, Sema4A may serve broader functions during dental axon growth as there is evidence that Sema4A signaling is involved in enhancing cortical neuron outgrowth and photoreceptor survival and phototransduction (Ishii et al., 1992).

Another member of the Sema4-family, namely Sema4D, is a potent axonal guidance factor both for nerves in the central and peripheral nervous system (Masuda et al., 2004) (Swiercz et al., 2002). Sema4D mRNAs were observed in the odontoblast layer before and after the first nerve fibers had arrived in the dentin-pulp border target area, and PlexinB1 and -B2, which are Sema4D signaling receptors, were found in postnatal trigeminal ganglion neurons. The findings that Sema4D transcripts are present in the trigeminal ganglion at E12 (Fazzari et al., 2007) and it can stimulate axonal branching and growth of E12.5 mouse sensory dorsal root ganglion neurons in an autocrine manner (Masuda et al., 2004), suggest that Sema4D acts as a neurite-outgrowth stimulating, autocrine/paracrine factor during development of the embryonic sensory neurons (Masuda et al., 2004). Because postnatal trigeminal ganglion neurons showed Sema4D immunoreaction it is possible that Sema4D may serve autocrine dental axon growth promoting functions during tooth innervation. Moreover, Sema4D may also regulate innervation of the pulp-dentin border target area, similarly as has been proposed for Sema7A in terminal innervation of the dentin-pulp complex (Maurin et al., 2005), possibly via a paracrine mode of action. On the other hand it has been reported that Sema4D is able to inhibit mature axon growth (Moreau-Fauvarque et al., 2003). It can also induce collapse of growth cones in CNS axons (Swiercz et al., 2002) and repel hippocampal and retinal neurons during development (Kruger et al., 2005) (Swiercz et al., 2002). Thus, it is possible that like Sema3A, Sema4D may exert an inhibitory influence and cause dental axonal growth cone collapse during tooth innervation.

Collectively, the data obtained regarding Sema3A and class 4 semaphorins suggest that semaphorin signaling may exert distinct and/or opposite effects on tooth innervation, possibly by autocrine and paracrine modes of action. Further investigation is required in order to further unravel the apparently complex functions of semaphorin signaling during development of the nerve supply in the tooth.

5.3 Sema3A appears not to serve non-neuronal functions during incisor tooth formation

Sema3A mRNAs were expressed in the incisor tooth germ at sites that did not show any apparent correlation with neural development such as in the outer dental epithelium, cervical loop and tooth-supporting developing alveolar bone (Moe et al., 2011). The epithelial cervical loop of the incisor contributes to the constant eruption of the tooth. The ameloblasts**,** which are responsible for production of enamel on the labial side of the incisor, are derived from the stem cells located in the cervical loop (Harada et al., 1999) (Klein et al., 2008) (Tummers and Thesleff, 2009) (Juuri et al., 2012). Earlier Sema3A has been shown to control various non-neuronal cellular processes that also take place during formation of the tooth and its surrounding tissues, such as cell proliferation, adhesion, cell death and patterning (Tran et al., 2007) (Yazdani and Terman, 2006) (Behar et al., 1996) (Hayashi et al., 2012). In particular, Sema3A-deficient mice suffer from abnormalities in bone formation and physiology, and exhibit defects such as partial rib duplications, vertebral fusions, osteopenia and defects in the heart (Behar et al., 1996) (Hayashi et al., 2012). Recently, it was suggested that Sema3A might even increase bone mass via sensory innervation (Fukuda et al., 2013). Different class 3 semaphorin receptors have been reported in the developing tooth (Loes et al., 2001) (Kettunen et al., 2005) (Perala et al., 2005) (Sijaona et al., 2012). Together these results raise the possibility that Sema3A might serve non-neuronal roles during odontogenesis, perhaps by even regulating epithelial dental stem cells during formation of tooth germ proper, as well as being involved in the development of tooth-supporting alveolar bone. Indeed, Sema3A has been proposed to be able to convert periodontal ligament cells of the human tooth into mesenchymal-stem-like cells (Wada et al., 2014).

Earlier, the cellular expression patterns of the neuroregulatory molecules in the developing tooth such as neurotrophins and Gdnf-family members have suggested that they might even serve non-neuronal functions during odontogenesis (Nosrat et al., 1997) (Luukko et al., 1997a) (Luukko et al., 1997b) (Luukko et al., 1996). Recently, Nerve growth factor was shown to promote differentiation of odontoblast-like cells (Arany et al., 2009). In the present study, however, no apparent abnormalities were observed in tooth histomorphogenesis, enamel, dentin or alveolar bone formation in the studied Sema3A-deficient incisor or molar tooth germs. This would indicate that Sema3A signaling appears not to serve major critical non-neuronal roles during tooth formation. This is in line with an earlier report showing no obvious defects in the early developing molar (Kettunen et al., 2005). In addition, PlexinA4 or -A2 receptor mRNAs were not reported in E14 molar tooth germ (Perala et al., 2005). It is thus possible that Sema3A may not serve a role in tooth formation or that its functions, if any, are redundant together with other signals expressed in the tooth such as other semaphorins reported in the developing tooth (Kettunen et al., 2005) (Loes et al., 2001) (Lillesaar and Fried, 2004) (Abe et al., 2008) (Korostylev et al., 2008) (Inagaki et al., 1995).

5.4 Class 4 semaphorin signaling in tooth formation

Sema4A mRNAs also showed a developmentally regulated cellular expression in the epithelial enamel organ of the molar tooth germ that did not show any apparent correlation with innervation. In particular, *Sema4A* mRNAs were observed in the inner dental epithelium and cervical loop, and later expression continued in the highly proliferative Hertwigs's epithelial root sheath (HERS) (Tummers et al., 2007). The expression of *Sema4A* in the dental epithelium during its growth and morphogenesis suggests that Sema4A might be involved in cell proliferation and thereby in the regulation of crown shape and subsequent root formation. Indeed, Plexin-B1 controls branching morphogenesis of kidney (Korostylev et al., 2008). Moreover, receptors for Sema4A, *PlexinB1* and *-B2* (Yukawa et al., 2005) were found in the dental epithelium during the stages studied, making it possible that signaling of Sema4A via PlexinB1, and possibly Plexin-B2 receptor, may be involved in tooth development.

Sema4A has been reported to suppress angiogenesis, and PlexinD1 receptor mRNAs were observed in blood vessels in the dental pulp (Toyofuku et al., 2007) (van der Zwaag et al., 2002). Therefore, it is possible that membrane-bound Sema4A, signaling through PlexinD1 may be involved in the regulation of tooth blood supply. mRNAs for PlexinB3, which mediates Sema4A signaling (Yukawa et al., 2010) showed no specific cellular expression domain in the postnatal molar tooth germ or neighboring tissues. This is in line with earlier studies showing that *PlexinB3* is not expressed in embryos, and later its expression is found only in oligodendrocytes (Perala et al., 2005) (Pasquale, 2000). This would suggest that Sema4A appears not to serve significant non-neuronal function(s) in the formation of the tooth and its surrounding tissues.

Differentiation of odonto- and ameloblasts, and subsequent dentin and enamel formation, takes place as a result of interactions between dental epithelium and mesenchyme and involves molecular signaling (Thesleff et al., 2001) (Coin et al., 1999) (Wang et al., 2004), including perhaps semaphorin signaling (Abe et al., 2008) (Sijaona et al., 2012). Expression of *Sema3F* has suggested a role in the differentiation of ameloblast cell lineage (Sijaona et al., 2012). Sema4D mRNAs were expressed in the preodontoblasts and later in the differentiated, dentin producing odontoblasts, and in the ameloblasts. An apparent expression of *Sema4D* was also observed in developing alveolar bone surrounding the tooth suggesting that bone-producing osteoblasts and bone-resorpting osteoclasts express this gene. Taken together this data suggest that Sema4D may be involved in the formation of the crown hard tissues and in tooth-supporting alveolar bone, possibly through autocrine and paracrine signaling. The putative role of Sema4D in regulation of dental hard tissue formation in tooth may be supported by the finding that overexpression of Sema4D negatively regulates type I collagen formation in dental pulp cells derived *in vitro* (Abe et al., 2008). Furthermore, osteoclast expressed Sema4D using PlexinB1 receptor in osteoblasts can suppress bone formation (Negishi-Koga et al., 2011). Expression of *Sema4C* in the shortened post-secretory ameloblasts suggests that Sema4C might be involved in regulation of the maturation of enamel.

The enamel organ of the developing tooth undergoes epithelial morphogenesis. Sema4D has also been shown to serve critical functions in organ morphogenesis. Sema4D inhibits kidney morphogenesis *in vitro* and epithelial branching morphogenesis of the kidney is defected in PlexinB1 deficient mice (Korostylev et al., 2008). Lack of Plexin-B2 results in decreased branching and proliferation of the ureteric epithelium and to small kidneys (Perala et al., 2011). It is therefore tempting to speculate that dental mesenchyme expressed Sema4D might bind to dental epithelium expressed PlexinB1 and/or -B2 receptors during embryogenesis (Perala et al., 2005) and perinatally and thereby mediate odontogenic epithelial-mesenchymal interactions and hence regulate different processes occurring in tooth formation such as in the morphogenesis of epithelial folding.

Class 4 semaphorin signaling has also been implicated in several infectious and non-infectious human diseases and processes such as the immune response (Nkyimbeng-Takwi and Chapoval, 2011). In particular, the neuroimmune semaphorins, Sema4A and Sema4D serve important roles in the immune response occurring in various diseases (Kumanogoh et al., 2002) (Kumanogoh et al., 2000) (Kumanogoh et al., 2005). T lymphocytes have been reported to be present in the dental pulp and show an increase after injury or following a noxious stimulus (Jontell et al., 1998). Antigen-presenting cells have been observed next to and in the odontoblast layer in the unerupted tooth and mainly in the subodontoblastic region in the erupted tooth suggesting that immunological defense potential is present in the dental pulp before tooth eruption (Yoshiba et al., 1996). Because Sema4A and Sema4D mRNAs were observed in dental pulp mesenchyme, it is possible that they might serve functions in the immune response in the dental pulp. Other tooth germ expressed semaphorins, which have been shown to have roles in immune system are for example Sema3A, -3C, -4B, -7A, PlexinA1, -A4, -B1, -B2, -C1, -D1 and Npn1 (Potiron et al., 2007) (Perala et al., 2012). It is apparent that further studies are needed to unravel the functions of semaphorin signaling and how its signaling pathways are integrated with other signaling pathways regulating organogenesis, tooth innervation and immune response.

6. CONCLUSIONS

In the present study it was found that Sema3A regulates developing incisor and postnatal molar innervation, and that expression patterns of class 4 semaphorins and their receptors suggest that they may serve neuronal and non-neuronal functions in developing molar tooth germ

-Sema3A mRNAs were found to show developmentally regulated cellular expression domains in the embryonic and postnatal mouse mandibular incisor tooth germ

-Sema3A regulates the innervation of the embryonic and postnatal developing incisor as well as innervation of the postnatal molar tooth

- Ngf, Gdnf and Ncam mRNAs were expressed in the postnatal Sema3A-deficient mouse incisor and molar. In addition, trkA, p75 and Ret, Npn1, PlexinA4 receptor mRNAs were expressed in the postnatal Sema3A-deficient trigeminal ganglion

-histo-morphology of the Sema3A-deficient incisor tooth germ, adult incisor and postnatal molar appeared normal

-Sema4A, -4C and -4D as well as PlexinB1 and -B2 showed distinct expression in the early postnatal developing mouse mandibular molar tooth germ. In addition, protein products of Sema4D, PlexinB1 and -B2 were present in the postnatal trigeminal ganglion

Taken together, the results obtained in this study provide further evidence that semaphorin signaling regulate tooth innervation. Moreover, semaphorin signaling may also serve non-neuronal functions both in developing and adult tooth.

7. FUTURE PERSPECTIVES

Tooth formation and development of the tooth-supporting peripheral sensory nerve supply are tightly integrated processes involving coordinated, developmentally regulated signaling events regulated by various signaling molecules. The results

obtained provide novel data regarding expression of Sema3A and selected class 4 semaphorin family members in the developing tooth and its supporting tissues, and functions of Sema3A in tooth innervation. Cellular expression domains of semaphorins that were not related to innervation suggest non-neuronal functions for semaphorin signaling in odontogenesis. However, no apparent morphological or histological changes were detected in the present studies on Sema3A-deficient teeth. It is considered that the obtained results provide significant data for future studies concerning the roles of semaphorin signaling during tooth formation including root development and eruption, and development of tooth supporting tissues. Further work such as more detailed developmental and neurobiological studies, including an investigation of various semaphorin signaling components in transgenic mouse models, is warranted in order to unravel other neuronal and putative non-neuronal functions of semaphorins in odontogenesis. In particular, regulatory molecular interactions occurring between different dental tissues such as epithelial and mesenchymal tissue components of the tooth organ proper and its supporting tissues, such as peripheral nerves, need to be studied. It is anticipated that future research will provide important basal information necessary for both discovering and developing novel approaches regarding biological treatment in clinical dentistry, such as restoration and regeneration of damaged dental pulp and periodontal tissues. It may even be possible to generate biological teeth or dental structures by applying emerging stem cell methods and nanotechnology.

8. REFERENCES

Abe M, Inagaki S, Furuyama T, Iwamoto M, Wakisaka S. 2008. Semaphorin 4D inhibits collagen synthesis of rat pulp-derived cells. Arch Oral Biol 53:27-34.

Adams RH, Lohrum M, Klostermann A, Betz H, Puschel AW. 1997. The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing. EMBO J 16:6077-6086.

Alkhamrah BA, Hoshino N, Kawano Y, Harada F, Hanada K, Maeda T. 2003. The periodontal Ruffini endings in brain derived neurotrophic factor (BDNF) deficient mice. Arch Histol Cytol 66:73-81.

Amar S, Karcher-Djuricic V, Meyer JM, Ruch JV. 1986. The lingual (root analogue) and the labial (crown analogue) mouse incisor dentin promotes ameloblast differentiation. Arch Anat Microsc Morphol Exp 75:229-239.

Arany S, Koyota S, Sugiyama T. 2009. Nerve growth factor promotes differentiation of odontoblast-like cells. J Cell Biochem 106:539-545.

Avery JK. 1994. Oral Development and Histology.

Bagnard D, Lohrum M, Uziel D, Puschel AW, Bolz J. 1998. Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. Development 125:5043-5053.

Barron MJ, McDonnell ST, Mackie I, Dixon MJ. 2008. Hereditary dentine disorders: dentinogenesis imperfecta and dentine dysplasia. Orphanet J Rare Dis 3:31.

Bashaw GJ, Klein R. 2010. Signaling from axon guidance receptors. Cold Spring Harb Perspect Biol 2:24.

Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC. 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383:525-528.

Bellon A, Luchino J, Haigh K, Rougon G, Haigh J, Chauvet S, Mann F. 2010. VEGFR2 (KDR/Flk1) signaling mediates axon growth in response to semaphorin 3E in the developing brain. Neuron 66:205-219.

Ben-Zvi A, Sweetat S, Behar O. 2013. Elimination of aberrant DRG circuitries in Sema3A mutant mice leads to extensive neuronal deficits. PLoS One 8.

Bluteau G, Luder HU, De Bari C, Mitsiadis TA. 2008. Stem cells for tooth engineering. Eur Cell Mater 16:1-9.

Bougeret C, Mansur IG, Dastot H, Schmid M, Mahouy G, Bensussan A, Boumsell L. 1992. Increased surface expression of a newly identified 150-kDa dimer early after human T lymphocyte activation. J Immunol 148:318-323.

Boukari A, Ruch JV. 1981. [Behavior of embryonic mouse teeth in vitro: preservation of the crown pattern and mineralization]. J Biol Buccale 9:349-361.

Bovolenta P. 2005. Morphogen signaling at the vertebrate growth cone: a few cases or a general strategy? J Neurobiol 64:405-416.

Byers MR. 1984. Dental sensory receptors. Int Rev Neurobiol 25:39-94.

Byers MR. 1994. Dynamic plasticity of dental sensory nerve structure and cytochemistry. Arch Oral Biol 39 Suppl:13s-21s.

Byers MR, Narhi MV. 1999. Dental injury models: experimental tools for understanding neuroinflammatory interactions and polymodal nociceptor functions. Crit Rev Oral Biol Med 10:4-39.

Byers MR, Suzuki H, Maeda T. 2003. Dental neuroplasticity, neuro-pulpal interactions, and nerve regeneration. Microsc Res Tech 60:503-515.
Byers MR, Wheeler EF, Bothwell M. 1992. Altered expression of NGF and P75 NGF-receptor by fibroblasts of injured teeth precedes sensory nerve sprouting. Growth Factors 6:41-52.
Charles C, Lazzari V, Tafforeau P, Schimmang T, Tekin M, Klein O, Viriot L. 2009. Modulation of Fgf3 dosage in mouse and men mirrors evolution of mammalian dentition. Proc Natl Acad Sci U S A 106:22364-22368.
Charron F, Tessier-Lavigne M. 2005. Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development 132:2251-2262.
Chauvet S, Cohen S, Yoshida Y, Fekrane L, Livet J, Gayet O, Segu L, Buhot MC, Jessell TM, Henderson CE, Mann F. 2007. Gating of Sema3E/PlexinD1 signaling by neuropilin-1 switches axonal repulsion to attraction during brain development. Neuron 56:807-822.
Chedotal A, Del Rio JA, Ruiz M, He Z, Borrell V, de Castro F, Ezan F, Goodman CS, Tessier-Lavigne M, Sotelo C, Soriano E. 1998. Semaphorins III and IV repel hippocampal axons via two distinct receptors. Development 125:4313-4323.
Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, Pleasure SJ, Lowenstein DH, Skarnes WC, Chedotal A, Tessier-Lavigne M. 2000. Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron 25:43-56.
Cheng HJ, Bagri A, Yaron A, Stein E, Pleasure SJ, Tessier-Lavigne M. 2001. Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. Neuron 32:249-263.
Chung L, Yang TL, Huang HR, Hsu SM, Cheng HJ, Huang PH. 2007. Semaphorin signaling facilitates cleft formation in the developing salivary gland. Development 134:2935-2945.
Cobourne MT, Sharpe PT. 2003. Tooth and jaw: molecular mechanisms of patterning in the first branchial arch. Arch Oral Biol 48:1-14.
Cobourne MT, Sharpe PT. 2010. Making up the numbers: The molecular control of mammalian dental formula. Semin Cell Dev Biol 21:314-324.
Coin R, Haikel Y, Ruch JV. 1999. Effects of apatite, transforming growth factor beta-1, bone morphogenetic protein-2 and interleukin-7 on ameloblast differentiation in vitro. Eur J Oral Sci 107:487-495.
Committee SN. 1999. Unified nomenclature for the semaphorins/collapsins. Cell 97:551-552.
Dassule HR, Lewis P, Bei M, Maas R, McMahon AP. 2000. Sonic hedgehog regulates growth and morphogenesis of the tooth. Development 127:4775-4785.
Davies AM. 1988. The trigeminal system: an advantageous experimental model for studying neuronal development. Development 103 Suppl:175-183.
Davies AM. 1997. Studies of neurotrophin biology in the developing trigeminal system. J Anat 191 (Pt 4):483-491.
Davies AM, Lumsden AG, Slavkin HC, Burnstock G. 1981. Influence of nerve growth factor on the embryonic mouse trigeminal ganglion in culture. Dev Neurosci 4:150-156.

Degenhardt K, Singh MK, Aghajanian H, Massera D, Wang Q, Li J, Li L, Choi C, Yzaguirre AD, Francey LJ, Gallant E, Krantz ID, Gruber PJ, Epstein JA. 2013. Semaphorin 3d signaling defects are associated with anomalous pulmonary venous connections. Nat Med 19:760-765.

Delaire S, Billard C, Tordjman R, Chedotal A, Elhabazi A, Bensussan A, Boumsell L. 2001. Biological activity of soluble CD100. II. Soluble CD100, similarly to H-SemaIII, inhibits immune cell migration. J Immunol 166:4348-4354.

Dent EW, Barnes AM, Tang F, Kalil K. 2004. Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. J Neurosci 24:3002-3012.

Derveaux S, Vandesompele J, Hellemans J. 2010. How to do successful gene expression analysis using real-time PCR. Methods 50:227-230.

Dillon TE, Saldanha J, Giger R, Verhaagen J, Rochlin MW. 2004. Sema3A regulates the timing of target contact by cranial sensory axons. J Comp Neurol 470:13-24.

Dontchev VD, Letourneau PC. 2002. Nerve growth factor and semaphorin 3A signaling pathways interact in regulating sensory neuronal growth cone motility. J Neurosci 22:6659-6669.

Dontchev VD, Letourneau PC. 2003. Growth cones integrate signaling from multiple guidance cues. J Histochem Cytochem 51:435-444.

Elhabazi A, Delaire S, Bensussan A, Boumsell L, Bismuth G. 2001. Biological activity of soluble CD100. I. The extracellular region of CD100 is released from the surface of T lymphocytes by regulated proteolysis. J Immunol 166:4341-4347.

Emmenlauer M, Ronneberger O, Ponti A, Schwarb P, Griffa A, Filippi A, Nitschke R, Driever W, Burkhardt H. 2009. XuvTools: free, fast and reliable stitching of large 3D datasets. J Microsc 233:42-60.

Encinas JA, Kikuchi K, Chedotal A, de Castro F, Goodman CS, Kimura T. 1999. Cloning, expression, and genetic mapping of Sema W, a member of the semaphorin family. Proc Natl Acad Sci U S A 96:2491-2496.

Erdelyi G, Fried K, Hildebrand C. 1987. Nerve growth to tooth buds after homotopic or heterotopic autotransplantation. Brain Res 430:39-47.

Falk J, Bechara A, Fiore R, Nawabi H, Zhou H, Hoyo-Becerra C, Bozon M, Rougon G, Grumet M, Puschel AW, Sanes JR, Castellani V. 2005. Dual functional activity of semaphorin 3B is required for positioning the anterior commissure. Neuron 48:63-75.

Fazzari P, Penachioni J, Gianola S, Rossi F, Eickholt BJ, Maina F, Alexopoulou L, Sottile A, Comoglio PM, Flavell RA, Tamagnone L. 2007. Plexin-B1 plays a redundant role during mouse development and in tumour angiogenesis. BMC Dev Biol 7:55.

Feiner L, Webber AL, Brown CB, Lu MM, Jia L, Feinstein P, Mombaerts P, Epstein JA, Raper JA. 2001. Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. Development 128:3061-3070.

Fried K, Erdelyi G. 1982. Inferior alveolar nerve regeneration and incisor pulpal reinnervation following intramandibular neurotomy in the cat. Brain Res 244:259-268.

Fried K, Lillesaar C, Sime W, Kaukua N, Patarroyo M. 2007. Target finding of pain nerve fibers: neural growth mechanisms in the tooth pulp. Physiol Behav 92:40-45.
Fried K, Nosrat C, Lillesaar C, Hildebrand C. 2000. Molecular signaling and pulpal nerve development. Crit Rev Oral Biol Med 11:318-332.
Fried K, Sime W, Lillesaar C, Virtanen I, Tryggvasson K, Patarroyo M. 2005. Laminins 2 (alpha2beta1gamma1, Lm-211) and 8 (alpha4beta1gamma1, Lm-411) are synthesized and secreted by tooth pulp fibroblasts and differentially promote neurite outgrowth from trigeminal ganglion sensory neurons. Exp Cell Res 307:329-341.
Fristad I, Heyeraas KJ, Kvinnsland I. 1994. Nerve fibres and cells immunoreactive to neurochemical markers in developing rat molars and supporting tissues. Arch Oral Biol 39:633-646.
Fuchs E, Chen T. 2013. A matter of life and death: self-renewal in stem cells. EMBO Rep 14:39-48.
Fujisawa H. 2003. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. J Neurobiol 59:24-33.
Fujiyama K, Yamashiro T, Fukunaga T, Balam TA, Zheng L, Takano-Yamamoto T. 2004. Denervation resulting in dento-alveolar ankylosis associated with decreased Malassez epithelium. J Dent Res 83:625-629.
Fukuda T, Takeda S, Xu R, Ochi H, Sunamura S, Sato T, Shibata S, Yoshida Y, Gu Z, Kimura A, Ma C, Xu C, Bando W, Fujita K, Shinomiya K, Hirai T, Asou Y, Enomoto M, Okano H, Okawa A, Itoh H. 2013. Sema3A regulates bone-mass accrual through sensory innervations. Nature 5.
Georgieff IS, Liem RK, Couchie D, Mavilia C, Nunez J, Shelanski ML. 1993. Expression of high molecular weight tau in the central and peripheral nervous systems. J Cell Sci 105 (Pt 3):729-737.
Gibson DA, Ma L. 2011. Developmental regulation of axon branching in the vertebrate nervous system. Development 138:183-195.
Giger RJ, Cloutier JF, Sahay A, Prinjha RK, Levengood DV, Moore SE, Pickering S, Simmons D, Rastan S, Walsh FS, Kolodkin AL, Ginty DD, Geppert M. 2000. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron 25:29-41.
Giger RJ, Hollis ER, 2nd, Tuszynski MH. 2010. Guidance molecules in axon regeneration. Cold Spring Harb Perspect Biol 2:a001867.
Gitler AD, Lu MM, Epstein JA. 2004. PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. Dev Cell 7:107-116.
Goodman CS, Kolodkin AL, Luo Y, Puschel AW, Raper JA, Comm SN. 1999. Unified nomenclature for the semaphorins collapsins. Cell 97:551-552.
Gritli-Linde A, Bei M, Maas R, Zhang XM, Linde A, McMahon AP. 2002. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. Development 129:5323-5337.
Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 97:13625-13630.

Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD. 2005a. Semaphorin 3E and Plexin-D1 Control Vascular Pattern Independently of Neuropilins. Science 307:265-268.
Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD. 2005b. Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. Science 307:265-268.
Guan F, Villegas G, Teichman J, Mundel P, Tufro A. 2006. Autocrine class 3 semaphorin system regulates slit diaphragm proteins and podocyte survival. Kidney Int 69:1564-1569.
Gurdon JB. 1992. The generation of diversity and pattern in animal development. Cell 68:185-199.
Handa K, Saito M, Tsunoda A, Yamauchi M, Hattori S, Sato S, Toyoda M, Teranaka T, Narayanan AS. 2002a. Progenitor cells from dental follicle are able to form cementum matrix in vivo. Connect Tissue Res 43:406-408.
Handa K, Saito M, Yamauchi M, Kiyono T, Sato S, Teranaka T, Sampath Narayanan A. 2002b. Cementum matrix formation in vivo by cultured dental follicle cells. Bone 31:606-611.
Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I. 1999. Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. J Cell Biol 147:105-120.
Harlow E, Lane D. 1999. Using antibodies: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. xiv, 495 s. : ill. pp.
Haug SR, Heyeraas KJ. 2006. Modulation of dental inflammation by the sympathetic nervous system. J Dent Res 85:488-495.
Haupt C, Kloos K, Faus-Kessler T, Huber AB. 2010. Semaphorin 3A-Neuropilin-1 signaling regulates peripheral axon fasciculation and pathfinding but not developmental cell death patterns. Eur J Neurosci 31:1164-1172.
Hay MF. 1961. The development in vivo and in vitro of the lower incisor and molars of the mouse. Arch Oral Biol 3:86-109.
Hayashi M, Nakashima T, Taniguchi M, Kodama T, Kumanogoh A, Takayanagi H. 2012. Osteoprotection by semaphorin 3A. Nature 485:69-74.
He Z, Tessier-Lavigne M. 1997. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90:739-751.
Herrmann H, Aebi U. 2000. Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. Curr Opin Cell Biol 12:79-90.
Heyeraas KJ, Berggreen E. 1999. Interstitial fluid pressure in normal and inflamed pulp. Crit Rev Oral Biol Med 10:328-336.
Hildebrand C, Fried K, Tuisku F, Johansson CS. 1995. Teeth and tooth nerves. Prog Neurobiol 45:165-222.
Holland GR, Robinson PP. 1985. Reinnervation of the canine tooth pulp after section of the inferior alveolar nerve in the cat. Brain Res 329:300-303.
Holland GR, Robinson PP. 1987. Pulp re-innervation in re-implanted canine teeth of the cat. Arch Oral Biol 32:593-597.

Huber AB, Kania A, Tran TS, Gu C, De Marco Garcia N, Lieberam I, Johnson D, Jessell TM, Ginty DD, Kolodkin AL. 2005. Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. Neuron 48:949-964.

Huber AB, Kolodkin AL, Ginty DD, Cloutier JF. 2003. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci 26:509-563.

Inagaki S, Furuyama T, Iwahashi Y. 1995. Identification of a member of mouse semaphorin family. FEBS Lett 370:269-272.

Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A. 2004. Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2. J Dent Res 83:590-595.

Ishii N, Wadsworth WG, Stern BD, Culotti JG, Hedgecock EM. 1992. UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in C. elegans. Neuron 9:873-881.

Ito T, Kagoshima M, Sasaki Y, Li C, Udaka N, Kitsukawa T, Fujisawa H, Taniguchi M, Yagi T, Kitamura H, Goshima Y. 2000. Repulsive axon guidance molecule Sema3A inhibits branching morphogenesis of fetal mouse lung. Mech Dev 97:35-45.

Ito Y, Oinuma I, Katoh H, Kaibuchi K, Negishi M. 2006. Sema4D/plexin-B1 activates GSK-3beta through R-Ras GAP activity, inducing growth cone collapse. EMBO Rep 7:704-709.

Jernvall J, Kettunen P, Karavanova I, Martin LB, Thesleff I. 1994. Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating Fgf-4 gene. Int J Dev Biol 38:463-469.

Jernvall J, Thesleff I. 2000. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 92:19-29.

Jernvall J, Thesleff I. 2012. Tooth shape formation and tooth renewal: evolving with the same signals. Development 139:3487-3497.

Jontell M, Okiji T, Dahlgren U, Bergenholtz G. 1998. Immune defense mechanisms of the dental pulp. Crit Rev Oral Biol Med 9:179-200.

Jussila M, Thesleff I. 2012. Signaling networks regulating tooth organogenesis and regeneration, and the specification of dental mesenchymal and epithelial cell lineages. Cold Spring Harb Perspect Biol 4.

Juuri E, Saito K, Ahtiainen L, Seidel K, Tummers M, Hochedlinger K, Klein OD, Thesleff I, Michon F. 2012. Sox2+ Stem Cells Contribute to All Epithelial Lineages of the Tooth via Sfrp5+ Progenitors. Dev Cell 23:317-328.

Kagoshima M, Ito T. 2001. Diverse gene expression and function of semaphorins in developing lung: positive and negative regulatory roles of semaphorins in lung branching morphogenesis. Genes Cells 6:559-571.

Kanda T, Yoshida Y, Izu Y, Nifuji A, Ezura Y, Nakashima K, Noda M. 2007. PlexinD1 deficiency induces defects in axial skeletal morphogenesis. J Cell Biochem 101:1329-1337.

Kawasaki T, Bekku Y, Suto F, Kitsukawa T, Taniguchi M, Nagatsu I, Nagatsu T, Itoh K, Yagi T, Fujisawa H. 2002. Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. Development 129:671-680.

Keranen SV, Kettunen P, Aberg T, Thesleff I, Jernvall J. 1999. Gene expression patterns associated with suppression of odontogenesis in mouse and vole diastema regions. Dev Genes Evol 209:495-506.
Kettunen P, Laurikkala J, Itaranta P, Vainio S, Itoh N, Thesleff I. 2000. Associations of FGF-3 and FGF-10 with signaling networks regulating tooth morphogenesis. Dev Dyn 219:322-332.
Kettunen P, Loes S, Furmanek T, Fjeld K, Kvinnsland IH, Behar O, Yagi T, Fujisawa H, Vainio S, Taniguchi M, Luukko K. 2005. Coordination of trigeminal axon navigation and patterning with tooth organ formation: epithelial-mesenchymal interactions, and epithelial Wnt4 and Tgfbeta1 regulate semaphorin 3a expression in the dental mesenchyme. Development 132:323-334.
Kettunen P, Spencer-Dene B, Furmanek T, Kvinnsland IH, Dickson C, Thesleff I, Luukko K. 2007. Fgfr2b mediated epithelial-mesenchymal interactions coordinate tooth morphogenesis and dental trigeminal axon patterning. Mech Dev 124:868-883.
Kettunen P, Thesleff I. 1998. Expression and function of FGFs-4, -8, and -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. Developmental Dynamics 211:256-268.
Kitsukawa T, Shimizu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T, Fujisawa H. 1997. Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron 19:995-1005.
Klein OD, Lyons DB, Balooch G, Marshall GW, Basson MA, Peterka M, Boran T, Peterkova R, Martin GR. 2008. An FGF signaling loop sustains the generation of differentiated progeny from stem cells in mouse incisors. Development 135:377-385.
Klein OD, Oberoi S, Huysseune A, Hovorakova M, Peterka M, Peterkova R. 2013. Developmental disorders of the dentition: an update. Am J Med Genet C Semin Med Genet 163C:318-332.
Klostermann A, Lohrum M, Adams RH, Puschel AW. 1998. The chemorepulsive activity of the axonal guidance signal semaphorin D requires dimerization. J Biol Chem 273:7326-7331.
Kobayashi H, Koppel AM, Luo Y, Raper JA. 1997. A role for collapsin-1 in olfactory and cranial sensory axon guidance. J Neurosci 17:8339-8352.
Kollar EJ, Baird GR. 1970. Tissue interactions in embryonic mouse tooth germs. II. The inductive role of the dental papilla. J Embryol Exp Morphol 24:173-186.
Kollar EJ, Lumsden AG. 1979. Tooth morphogenesis: the role of the innervation during induction and pattern formation. J Biol Buccale 7:49-60.
Kolodkin AL. 1996. Semaphorins: mediators of repulsive growth cone guidance. Trends Cell Biol 6:15-22.
Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. 1997. Neuropilin is a semaphorin III receptor. Cell 90:753-762.
Kolodkin AL, Matthes DJ, Goodman CS. 1993. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 75:1389-1399.
Kolodkin AL, Tessier-Lavigne M. 2011. Mechanisms and molecules of neuronal wiring: a primer. Cold Spring Harb Perspect Biol 3.

Koppel AM, Feiner L, Kobayashi H, Raper JA. 1997. A 70 amino acid region within the semaphorin domain activates specific cellular response of semaphorin family members. Neuron 19:531-537.
Koppel AM, Raper JA. 1998. Collapsin-1 covalently dimerizes, and dimerization is necessary for collapsing activity. J Biol Chem 273:15708-15713.
Korostylev A, Worzfeld T, Deng S, Friedel RH, Swiercz JM, Vodrazka P, Maier V, Hirschberg A, Ohoka Y, Inagaki S, Offermanns S, Kuner R. 2008. A functional role for semaphorin 4D/plexin B1 interactions in epithelial branching morphogenesis during organogenesis. Development 135:3333-3343.
Kratochwil K, Galceran J, Tontsch S, Roth W, Grosschedl R. 2002. FGF4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in Lef1(-/-) mice. Genes Dev 16:3173-3185.
Kruger RP, Aurandt J, Guan KL. 2005. Semaphorins command cells to move. Nat Rev Mol Cell Biol 6:789-800.
Kumanogoh A, Kikutani H. 2013. Immunological functions of the neuropilins and plexins as receptors for semaphorins. Nature Reviews Immunology 13:802-814.
Kumanogoh A, Marukawa S, Suzuki K, Takegahara N, Watanabe C, Ch'ng E, Ishida I, Fujimura H, Sakoda S, Yoshida K, Kikutani H. 2002. Class IV semaphorin Sema4A enhances T-cell activation and interacts with Tim-2. Nature 419:629-633.
Kumanogoh A, Shikina T, Suzuki K, Uematsu S, Yukawa K, Kashiwamura S, Tsutsui H, Yamamoto M, Takamatsu H, Ko-Mitamura EP, Takegahara N, Marukawa S, Ishida I, Morishita H, Prasad DV, Tamura M, Mizui M, Toyofuku T, Akira S, Takeda K, Okabe M, Kikutani H. 2005. Nonredundant roles of Sema4A in the immune system: defective T cell priming and Th1/Th2 regulation in Sema4A-deficient mice. Immunity 22:305-316.
Kumanogoh A, Watanabe C, Lee I, Wang X, Shi W, Araki H, Hirata H, Iwahori K, Uchida J, Yasui T, Matsumoto M, Yoshida K, Yakura H, Pan C, Parnes JR, Kikutani H. 2000. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. Immunity 13:621-631.
Kurien BT, Scofield RH. 2006. Western blotting. Methods 38:283-293.
Kutschera S, Weber H, Weick A, De Smet F, Genove G, Takemoto M, Prahst C, Riedel M, Mikelis C, Baulande S, Champseix C, Kummerer P, Conseiller E, Multon MC, Heroult M, Bicknell R, Carmeliet P, Betsholtz C, Augustin HG. 2011. Differential endothelial transcriptomics identifies semaphorin 3G as a vascular class 3 semaphorin. Arterioscler Thromb Vasc Biol 31:151-159.
Kvinnsland I, Heyeraas KJ. 1992. Effect of traumatic occlusion on CGRP and SP immunoreactive nerve fibre morphology in rat molar pulp and periodontium. Histochemistry 97:111-120.
Kvinnsland IH, Luukko K, Fristad I, Kettunen P, Jackson DL, Fjeld K, Von Bartheld CS, Byers MR. 2004. Glial cell line-derived neurotrophic factor (GDNF) from adult rat tooth serves a distinct population of large-sized trigeminal neurons. Eur J Neurosci 19:2089-2098.
Lallier TE. 2004. Semaphorin Profiling of Periodontal Fibroblasts and Osteoblasts. Journal of Dental Research 83:677-682.

Lammi L, Arte S, Somer M, Jarvinen H, Lahermo P, Thesleff I, Pirinen S, Nieminen P. 2004. Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. Am J Hum Genet 74:1043-1050.
Lauria G, Borgna M, Morbin M, Lombardi R, Mazzoleni G, Sghirlanzoni A, Pareyson D. 2004. Tubule and neurofilament immunoreactivity in human hairy skin: markers for intraepidermal nerve fibers. Muscle Nerve 30:310-316.
Lequin RM. 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin Chem 51:2415-2418.
Lesot H, Brook AH. 2009. Epithelial histogenesis during tooth development. Arch Oral Biol 54 Suppl 1:S25-33.
Lesot H, Lisi S, Peterkova R, Peterka M, Mitolo V, Ruch JV. 2001. Epigenetic signals during odontoblast differentiation. Adv Dent Res 15:8-13.
Lewin GR, Barde YA. 1996. Physiology of the neurotrophins. Annu Rev Neurosci 19:289-317.
Li H, Wu DK, Sullivan SL. 1999. Characterization and expression of sema4g, a novel member of the semaphorin gene family. Mech Dev 87:169-173.
Lillesaar C, Arenas E, Hildebrand C, Fried K. 2003. Responses of rat trigeminal neurones to dental pulp cells or fibroblasts overexpressing neurotrophic factors in vitro. Neuroscience 119:443-451.
Lillesaar C, Eriksson C, Johansson CS, Fried K, Hildebrand C. 1999. Tooth pulp tissue promotes neurite outgrowth from rat trigeminal ganglia in vitro. J Neurocytol 28:663-670.
Lillesaar C, Fried K. 2004. Neurites from trigeminal ganglion explants grown in vitro are repelled or attracted by tooth-related tissues depending on developmental stage. Neuroscience 125:149-161.
Liu B, Chen S, Cheng D, Jing W, Helms JA. 2014. Primary cilia integrate hedgehog and Wnt signaling during tooth development. J Dent Res 93:475-482.
Liu Y, Halloran MC. 2005. Central and peripheral axon branches from one neuron are guided differentially by Semaphorin3D and transient axonal glycoprotein-1. J Neurosci 25:10556-10563.
Loes S, Kettunen P, Kvinnsland H, Luukko K. 2002. Mouse rudimentary diastema tooth primordia are devoid of peripheral nerve fibers. Anat Embryol (Berl) 205:187-191.
Loes S, Kettunen P, Kvinnsland IH, Taniguchi M, Fujisawa H, Luukko K. 2001. Expression of class 3 semaphorins and neuropilin receptors in the developing mouse tooth. Mech Dev 101:191-194.
Loes S, Luukko K, Hals Kvinnsland I, Salminen M, Kettunen P. 2003. Developmentally regulated expression of Netrin-1 and -3 in the embryonic mouse molar tooth germ. Dev Dyn 227:573-577.
Lumsden AG. 1982. The developing innervation of the lower jaw and its relation to the formation of tooth germs in mouse embryo. In: Kurten B, editor. Teeth : form, function and evolution : 5th International symposium on dentistry and morphology : Papers. New York ; Guildford: Columbia University Press. pp ix, 393 p.

Lumsden AG. 1988. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. Development 103 Suppl:155-169.
Luo Y, Raible D, Raper JA. 1993. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75:217-227.
Luukko K. 1997. Immunohistochemical localization of nerve fibres during development of embryonic rat molar using peripherin and protein gene product 9.5 antibodies. Arch Oral Biol 42:189-195.
Luukko K, Arumae U, Karavanov A, Moshnyakov M, Sainio K, Sariola H, Saarma M, Thesleff I. 1997a. Neurotrophin mRNA expression in the developing tooth suggests multiple roles in innervation and organogenesis. Dev Dyn 210:117-129.
Luukko K, Kettunen P. 2014. Coordination of tooth morphogenesis and neuronal development through tissue interactions: Lessons from mouse models. Exp Cell Res.
Luukko K, Kvinnsland IH, Kettunen P. 2005a. Tissue interactions in the regulation of axon pathfinding during tooth morphogenesis. Dev Dyn 234:482-488.
Luukko K, Loes S, Furmanek T, Fjeld K, Kvinnsland IH, Kettunen P. 2003. Identification of a novel putative signaling center, the tertiary enamel knot in the postnatal mouse molar tooth. Mech Dev 120:270-276.
Luukko K, Loes S, Kvinnsland IH, Kettunen P. 2005b. Expression of ephrin-A ligands and EphA receptors in the developing mouse tooth and its supporting tissues. Cell Tissue Res 319:143-152.
Luukko K, Moe K, Sijaona A, Furmanek T, Hals Kvinnsland I, Midtbo M, Kettunen P. 2008. Secondary induction and the development of tooth nerve supply. Ann Anat 190:178-187.
Luukko K, Moshnyakov M, Sainio K, Saarma M, Sariola H, Thesleff I. 1996. Expression of neurotrophin receptors during rat tooth development is developmentally regulated, independent of innervation, and suggests functions in the regulation of morphogenesis and innervation. Dev Dyn 206:87-99.
Luukko K, Saarma M, Thesleff I. 1998. Neurturin mRNA expression suggests roles in trigeminal innervation of the first branchial arch and in tooth formation. Dev Dyn 213:207-219.
Luukko K, Suvanto P, Saarma M, Thesleff I. 1997b. Expression of GDNF and its receptors in developing tooth is developmentally regulated and suggests multiple roles in innervation and organogenesis. Dev Dyn 210:463-471.
Madduri S, Papaloizos M, Gander B. 2009. Synergistic effect of GDNF and NGF on axonal branching and elongation in vitro. Neurosci Res 65:88-97.
Maeda T, Ochi K, Nakakura-Ohshima K, Youn SH, Wakisaka S. 1999. The Ruffini ending as the primary mechanoreceptor in the periodontal ligament: its morphology, cytochemical features, regeneration, and development. Crit Rev Oral Biol Med 10:307-327.
Maestrini E, Tamagnone L, Longati P, Cremona O, Gulisano M, Bione S, Tamanini F, Neel BG, Toniolo D, Comoglio PM. 1996. A family of transmembrane proteins with homology to the MET-hepatocyte growth factor receptor. Proc Natl Acad Sci U S A 93:674-678.

Magloire H, Maurin JC, Couble ML, Shibukawa Y, Tsumura M, Thivichon-Prince B, Bleicher F. 2010. Topical review. Dental pain and odontoblasts: facts and hypotheses. J Orofac Pain 24:335-349.
Maier V, Jolicoeur C, Rayburn H, Takegahara N, Kumanogoh A, Kikutani H, Tessier-Lavigne M, Wurst W, Friedel RH. 2011. Semaphorin 4C and 4G are ligands of Plexin-B2 required in cerebellar development. Molecular and Cellular Neuroscience 46:419-431.
Mark MD, Lohrum M, Puschel AW. 1997. Patterning neuronal connections by chemorepulsion: the semaphorins. Cell Tissue Res 290:299-306.
Marsick BM, Flynn KC, Santiago-Medina M, Bamburg JR, Letourneau PC. 2010. Activation of ADF/cofilin mediates attractive growth cone turning toward nerve growth factor and netrin-1. Dev Neurobiol 70:565-588.
Martin A, Unda FJ, Begue-Kirn C, Ruch JV, Arechaga J. 1998. Effects of aFGF, bFGF, TGFbeta1 and IGF-I on odontoblast differentiation in vitro. Eur J Oral Sci 106 Suppl 1:117-121.
Maruyama Y, Harada F, Jabbar S, Saito I, Aita M, Kawano Y, Suzuki A, Nozawa-Inoue K, Maeda T. 2005. Neurotrophin-4/5-depletion induces a delay in maturation of the periodontal Ruffini endings in mice. Arch Histol Cytol 68:267-288.
Masuda K, Furuyama T, Takahara M, Fujioka S, Kurinami H, Inagaki S. 2004. Sema4D stimulates axonal outgrowth of embryonic DRG sensory neurones. Genes Cells 9:821-829.
Matsuo S, Ichikawa H, Henderson TA, Silos-Santiago I, Barbacid M, Arends JJ, Jacquin MF. 2001. trkA modulation of developing somatosensory neurons in oro-facial tissues: tooth pulp fibers are absent in trkA knockout mice. Neuroscience 105:747-760.
Matsuo S, Ichikawa H, Silos-Santiago I, Kiyomiya K, Kurebe M, Arends JJ, Jacquin MF. 2002. Ruffini endings are absent from the periodontal ligament of trkB knockout mice. Somatosens Mot Res 19:213-217.
Maurin JC, Couble ML, Didier-Bazes M, Brisson C, Magloire H, Bleicher F. 2004. Expression and localization of reelin in human odontoblasts. Matrix Biol 23:277-285.
Maurin JC, Delorme G, Machuca-Gayet I, Couble ML, Magloire H, Jurdic P, Bleicher F. 2005. Odontoblast expression of semaphorin 7A during innervation of human dentin. Matrix Biol 24:232-238.
Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL. 1995. Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron 14:949-959.
Michon F, Tummers M, Kyyronen M, Frilander MJ, Thesleff I. 2010. Tooth morphogenesis and ameloblast differentiation are regulated by micro-RNAs. Dev Biol 340:355-368.
Miletich I, Sharpe PT. 2003. Normal and abnormal dental development. Hum Mol Genet 12 Spec No 1:R69-73.
Mina M, Kollar EJ. 1987. The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. Arch Oral Biol 32:123-127.

Mitsiadis TA, Couble P, Dicou E, Rudkin BB, Magloire H. 1993. Patterns of nerve growth factor (NGF), proNGF, and p75 NGF receptor expression in the rat incisor: comparison with expression in the molar. Differentiation 54:161-175.
Mitsiadis TA, Dicou E, Joffre A, Magloire H. 1992. Immunohistochemical localization of nerve growth factor (NGF) and NGF receptor (NGF-R) in the developing first molar tooth of the rat. Differentiation 49:47-61.
Mitsiadis TA, Luder HU. 2011. Genetic basis for tooth malformations: from mice to men and back again. Clin Genet 80:319-329.
Mitsiadis TA, Luukko K. 1995. Neurotrophins in odontogenesis. Int J Dev Biol 39:195-202.
Mitsiadis TA, Woloszyk A, Jimenez-Rojo L. 2012. Nanodentistry: combining nanostructured materials and stem cells for dental tissue regeneration. Nanomedicine (Lond) 7:1743-1753.
Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. 2003. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 100:5807-5812.
Moe K, Kettunen P, Kvinnsland IH, Luukko K. 2008. Development of the pioneer sympathetic innervation into the dental pulp of the mouse mandibular first molar. Arch Oral Biol 53:865-873.
Moe K, Shrestha A, Kvinnsland IH, Luukko K, Kettunen P. 2011. Developmentally regulated expression of Sema3A chemorepellant in the developing mouse incisor. Acta Odontol Scand.
Mohamed SS, Atkinson ME. 1983. A histological study of the innervation of developing mouse teeth. J Anat 136:735-749.
Moreau-Fauvarque C, Kumanogoh A, Camand E, Jaillard C, Barbin G, Boquet I, Love C, Jones EY, Kikutani H, Lubetzki C, Dusart I, Chedotal A. 2003. The transmembrane semaphorin Sema4D/CD100, an inhibitor of axonal growth, is expressed on oligodendrocytes and upregulated after CNS lesion. J Neurosci 23:9229-9239.
Mueller BK. 1999. Growth cone guidance: first steps towards a deeper understanding. Annu Rev Neurosci 22:351-388.
Naftel JP, Qian XB, Bernanke JM. 1994. Effects of postnatal anti-nerve growth factor serum exposure on development of apical nerves of the rat molar. Brain Res Dev Brain Res 80:54-62.
Naftel JP, Richards LP, Pan M, Bernanke JM. 1999. Course and composition of the nerves that supply the mandibular teeth of the rat. Anat Rec 256:433-447.
Nakagawa Y, Takamatsu H, Okuno T, Kang S, Nojima S, Kimura T, Kataoka TR, Ikawa M, Toyofuku T, Katayama I, Kumanogoh A. 2011. Identification of semaphorin 4B as a negative regulator of basophil-mediated immune responses. J Immunol 186:2881-2888.
Nakashima M. 1994. Induction of dentin formation on canine amputated pulp by recombinant human bone morphogenetic proteins (BMP)-2 and -4. J Dent Res 73:1515-1522.
Nanci A, Ten Cate AR. 2013. Ten Cate's oral histology : development, structure, and function. St. Louis, Mo.: Elsevier. p. p.

Negishi-Koga T, Shinohara M, Komatsu N, Bito H, Kodama T, Friedel RH, Takayanagi H. 2011. Suppression of bone formation by osteoclastic expression of semaphorin 4D. Nat Med 17:1473-1480.
Nelson SJ, Ash MM, Ash MM. 2010. Wheeler's dental anatomy, physiology, and occlusion. St. Louis, Mo.: Saunders/Elsevier. xvi, 346 p. pp.
Neufeld SJ, Zhou X, Vize PD, Cobb J. 2013. mRNA fluorescence in situ hybridization to determine overlapping gene expression in whole-mount mouse embryos. Dev Dyn 242:1094-1100.
Nieminen P. 2009. Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol 312B:320-342.
Nkyimbeng-Takwi E, Chapoval SP. 2011. Biology and function of neuroimmune semaphorins 4A and 4D. Immunol Res 50:10-21.
Nosrat A, Ryul Kim J, Verma P, P SC. 2014. Tissue Engineering Considerations in Dental Pulp Regeneration. Iran Endod J 9:30-39.
Nosrat CA, Fried K, Ebendal T, Olson L. 1998. NGF, BDNF, NT3, NT4 and GDNF in tooth development. Eur J Oral Sci 106 Suppl 1:94-99.
Nosrat CA, Fried K, Lindskog S, Olson L. 1997. Cellular expression of neurotrophin mRNAs during tooth development. Cell Tissue Res 290:569-580.
Nosrat CA, Tomac A, Lindqvist E, Lindskog S, Humpel C, Strömberg I, Ebendal T, Hoffer BJ, Olson L. 1996. Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. Cell and Tissue Research 286:191-207.
Nosrat IV, Smith CA, Mullally P, Olson L, Nosrat CA. 2004. Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons in vitro; implications for tissue engineering and repair in the nervous system. Eur J Neurosci 19:2388-2398.
O'Farrell PH. 1975. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007-4021.
Obara N, Takeda M. 1989. Innervation of mouse molars during the early states of tooth germ development. Higashi Nippon Shigaku Zasshi 8:115-124.
Obara N, Takeda M. 1993. Expression of neural cell adhesion molecule (NCAM) during the first molar development in the mouse. Anat Embryol (Berl) 187:209-219.
Ohshima H, Nakakura-Ohshima K, Yamamoto H, Maeda T. 2001. Alteration in the expression of heat shock protein (Hsp) 25-immunoreactivity in the dental pulp of rat molars following tooth replantation. Arch Histol Cytol 64:425-437.
Olgart L. 1996. Neural control of pulpal blood flow. Crit Rev Oral Biol Med 7:159-171.
Parysek LM, Goldman RD. 1988. Distribution of a novel 57 kDa intermediate filament (IF) protein in the nervous system. J Neurosci 8:555-563.
Pasquale E. 2000. Neurobiology. Turning attraction into repulsion. Science 289:1308-1310.
Patel TD, Jackman A, Rice FL, Kucera J, Snider WD. 2000. Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. Neuron 25:345-357.
Pearson AA. 1977. The early innervation of the developing deciduous teeth. J Anat 123:563-577.

Perala N, Jakobson M, Ola R, Fazzari P, Penachioni JY, Nymark M, Tanninen T, Immonen T, Tamagnone L, Sariola H. 2011. Sema4C-Plexin B2 signalling modulates ureteric branching in developing kidney. Differentiation 81:81-91.
Perala N, Sariola H, Immonen T. 2012. More than nervous: the emerging roles of plexins. Differentiation 83:77-91.
Perala NM, Immonen T, Sariola H. 2005. The expression of plexins during mouse embryogenesis. Gene Expr Patterns 5:355-362.
Potiron V, Nasarre P, Roche J, Healy C, Boumsell L. 2007. Semaphorin signaling in the immune system. Adv Exp Med Biol 600:132-144.
Puelles L. 2009. Contributions to Neuroembryology of Santiago Ramon y Cajal (1852-1934) and Jorge F. Tello (1880-1958). Int J Dev Biol 53:1145-1160.
Puschel AW, Adams RH, Betz H. 1995. Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron 14:941-948.
Qian XB, Naftel JP. 1996. Effects of neonatal exposure to anti-nerve growth factor on the number and size distribution of trigeminal neurones projecting to the molar dental pulp in rats. Arch Oral Biol 41:359-367.
Raper JA. 2000. Semaphorins and their receptors in vertebrates and invertebrates. Curr Opin Neurobiol 10:88-94.
Renaud J, Kerjan G, Sumita I, Zagar Y, Georget V, Kim D, Fouquet C, Suda K, Sanbo M, Suto F, Ackerman SL, Mitchell KJ, Fujisawa H, Chedotal A. 2008. Plexin-A2 and its ligand, Sema6A, control nucleus-centrosome coupling in migrating granule cells. Nat Neurosci 11:440-449.
Reue K. 1998. mRNA quantitation techniques: considerations for experimental design and application. J Nutr 128:2038-2044.
Rice DS, Huang W, Jones HA, Hansen G, Ye GL, Xu N, Wilson EA, Troughton K, Vaddi K, Newton RC, Zambrowicz BP, Sands AT. 2004. Severe retinal degeneration associated with disruption of semaphorin 4A. Invest Ophthalmol Vis Sci 45:2767-2777.
Robert C. 2010. Microarray analysis of gene expression during early development: a cautionary overview. Reproduction 140:787-801.
Rochlin MW, O'Connor R, Giger RJ, Verhaagen J, Farbman AI. 2000. Comparison of neurotrophin and repellent sensitivities of early embryonic geniculate and trigeminal axons. J Comp Neurol 422:579-593.
Rodrigues HG, Renaud S, Charles C, Le Poul Y, Sole F, Aguilar JP, Michaux J, Tafforeau P, Headon D, Jernvall J, Viriot L. 2013. Roles of dental development and adaptation in rodent evolution. Nat Commun 4.
Roth L, Koncina E, Satkauskas S, Cremel G, Aunis D, Bagnard D. 2009. The many faces of semaphorins: from development to pathology. Cell Mol Life Sci 66:649-666.
Ruch JV, Lesot H, Begue-Kirn C. 1995. Odontoblast differentiation. Int J Dev Biol 39:51-68.
Rutherford RB, Spangberg L, Tucker M, Rueger D, Charette M. 1994. The time-course of the induction of reparative dentine formation in monkeys by recombinant human osteogenic protein-1. Arch Oral Biol 39:833-838.

Sahay A, Molliver ME, Ginty DD, Kolodkin AL. 2003. Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. J Neurosci 23:6671-6680.
Salazar-Ciudad I, Jernvall J. 2002. A gene network model accounting for development and evolution of mammalian teeth. Proc Natl Acad Sci U S A 99:8116-8120.
Sanford SD, Gatlin JC, Hokfelt T, Pfenninger KH. 2008. Growth cone responses to growth and chemotropic factors. Eur J Neurosci 28:268-278.
Sarram S, Lee KF, Byers MR. 1997. Dental innervation and CGRP in adult p75-deficient mice. J Comp Neurol 385:297-308.
Sato O, Maeda T, Kobayashi S, Iwanaga T, Fujita T, Takahashi Y. 1988. Innervation of periodontal ligament and dental pulp in the rat incisor: an immunohistochemical investigation of neurofilament protein and glia-specific S-100 protein. Cell Tissue Res 251:13-21.
Schluter OM, Schmitz F, Jahn R, Rosenmund C, Sudhof TC. 2004. A complete genetic analysis of neuronal Rab3 function. J Neurosci 24:6629-6637.
Schonfeld SE, Slavkin HC. 1977. Demonstration of enamel matrix proteins on root-analogue surfaces of rabbit permanent incisor teeth. Calcif Tissue Res 24:223-229.
Schwarz Q, Waimey KE, Golding M, Takamatsu H, Kumanogoh A, Fujisawa H, Cheng HJ, Ruhrberg C. 2008. Plexin A3 and plexin A4 convey semaphorin signals during facial nerve development. Dev Biol 324:1-9.
Sharma A, Verhaagen J, Harvey AR. 2012. Receptor complexes for each of the Class 3 Semaphorins. Front Cell Neurosci 6:28.
Sharpe PT. 1995. Homeobox genes and orofacial development. Connect Tissue Res 32:17-25.
Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S. 2005. The efficacy of mesenchymal stem cells to regenerate and repair dental structures. Orthod Craniofac Res 8:191-199.
Shinmura Y, Tsuchiya S, Hata K, Honda MJ. 2008. Quiescent epithelial cell rests of Malassez can differentiate into ameloblast-like cells. J Cell Physiol 217:728-738.
Sijaona A, Luukko K, Kvinnsland IH, Kettunen P. 2012. Expression patterns of Sema3F, PlexinA4, -A3, Neuropilin1 and -2 in the postnatal mouse molar suggest roles in tooth innervation and organogenesis. Acta Odontol Scand 70:140-148.
Sire JY, Delgado SC, Girondot M. 2008. Hen's teeth with enamel cap: from dream to impossibility. BMC Evol Biol 8:246.
Six N, Lasfargues JJ, Goldberg M. 2002. Differential repair responses in the coronal and radicular areas of the exposed rat molar pulp induced by recombinant human bone morphogenetic protein 7 (osteogenic protein 1). Arch Oral Biol 47:177-187.
Skaliora I, Singer W, Betz H, Puschel AW. 1998. Differential patterns of semaphorin expression in the developing rat brain. Eur J Neurosci 10:1215-1229.
Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S. 2006. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One 1:e79.
Steindorff MM, Lehl H, Winkel A, Stiesch M. 2014. Innovative approaches to regenerate teeth by tissue engineering. Arch Oral Biol 59:158-166.

Stockton DW, Das P, Goldenberg M, D'Souza RN, Patel PI. 2000. Mutation of PAX9 is associated with oligodontia. Nat Genet 24:18-19.
Streit S, Michalski CW, Erkan M, Kleeff J, Friess H. 2009. Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. Nat Protoc 4:37-43.
Sutton AL, Zhang X, Dowd DR, Kharode YP, Komm BS, Macdonald PN. 2008. Semaphorin 3B is a 1,25-Dihydroxyvitamin D3-induced gene in osteoblasts that promotes osteoclastogenesis and induces osteopenia in mice. Mol Endocrinol 22:1370-1381.
Swiercz JM, Kuner R, Behrens J, Offermanns S. 2002. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. Neuron 35:51-63.
Szalay FS, Seligsohn D. 1977. Why did the strepsirhine tooth comb evolve. Folia Primatol (Basel) 27:75-82.
Takahashi T, Fournier A, Nakamura F, Wang LH, Murakami Y, Kalb RG, Fujisawa H, Strittmatter SM. 1999. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell 99:59-69.
Takamori K, Hosokawa R, Xu X, Deng X, Bringas P, Jr., Chai Y. 2008. Epithelial fibroblast growth factor receptor 1 regulates enamel formation. J Dent Res 87:238-243.
Takegahara N, Takamatsu H, Toyofuku T, Tsujimura T, Okuno T, Yukawa K, Mizui M, Yamamoto M, Prasad DV, Suzuki K, Ishii M, Terai K, Moriya M, Nakatsuji Y, Sakoda S, Sato S, Akira S, Takeda K, Inui M, Takai T, Ikawa M, Okabe M, Kumanogoh A, Kikutani H. 2006. Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. Nat Cell Biol 8:615-622.
Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM. 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99:71-80.
Tamagnone L, Comoglio PM. 2000. Signalling by semaphorin receptors: cell guidance and beyond. Trends in Cell Biology 10:377-383.
Tamagnone L, Comoglio PM. 2004. To move or not to move? Semaphorin signalling in cell migration. EMBO Rep 5:356-361.
Taniguchi M, Yuasa S, Fujisawa H, Naruse I, Saga S, Mishina M, Yagi T. 1997. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron 19:519-530.
Tenkova TI, Goldberg MP. 2007. A modified silver technique (de Olmos stain) for assessment of neuronal and axonal degeneration. Methods Mol Biol 399:31-39.
Tessier-Lavigne M, Goodman CS. 1996. The molecular biology of axon guidance. Science 274:1123-1133.
Thesleff I. 2003. Epithelial-mesenchymal signalling regulating tooth morphogenesis. J Cell Sci 116:1647-1648.
Thesleff I, Hurmerinta K. 1981. Tissue interactions in tooth development. Differentiation 18:75-88.

Thesleff I, Jarvinen E, Suomalainen M. 2007. Affecting tooth morphology and renewal by fine-tuning the signals mediating cell and tissue interactions. Novartis Found Symp 284:142-153; discussion 153-163.
Thesleff I, Jernvall J. 1997. The enamel knot: a putative signaling center regulating tooth development. Cold Spring Harb Symp Quant Biol 62:257-267.
Thesleff I, Keranen S, Jernvall J. 2001. Enamel knots as signaling centers linking tooth morphogenesis and odontoblast differentiation. Adv Dent Res 15:14-18.
Thesleff I, Nieminen P. 1996. Tooth morphogenesis and cell differentiation. Curr Opin Cell Biol 8:844-850.
Thesleff I, Vaahtokari A, Kettunen P, Aberg T. 1995a. Eplthelial-Mesenchymal Signaling during Tooth Development. Connective Tissue Research 32:9-15.
Thesleff I, Vaahtokari A, Partanen AM. 1995b. Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. Int J Dev Biol 39:35-50.
Thomas BL, Tucker AS, Qui M, Ferguson CA, Hardcastle Z, Rubenstein JL, Sharpe PT. 1997. Role of Dlx-1 and Dlx-2 genes in patterning of the murine dentition. Development 124:4811-4818.
Torres-Fernández O. 2006. [The Golgi silver impregnation method: commemorating the centennial of the Nobel Prize in medicine (1906) shared by Camillo Golgi and Santiago Ramon y Cajal]. Biomedica : revista del Instituto Nacional de Salud 26:498-508.
Torres-Vázquez J, Gitler AD, Fraser SD, Berk JD, Van NP, Fishman MC, Childs S, Epstein JA, Weinstein BM. 2004. Semaphorin-Plexin Signaling Guides Patterning of the Developing Vasculature. Developmental Cell 7:117-123.
Toyofuku T, Yabuki M, Kamei J, Kamei M, Makino N, Kumanogoh A, Hori M. 2007. Semaphorin-4A, an activator for T-cell-mediated immunity, suppresses angiogenesis via Plexin-D1. EMBO J 26:1373-1384.
Tran TS, Kolodkin AL, Bharadwaj R. 2007. Semaphorin regulation of cellular morphology. Annu Rev Cell Dev Biol 23:263-292.
Tsuzuki H, Kitamura H. 1991. Immunohistochemical analysis of pulpal innervation in developing rat molars. Arch Oral Biol 36:139-146.
Tucker A, Sharpe P. 2004. The cutting-edge of mammalian development; how the embryo makes teeth. Nat Rev Genet 5:499-508.
Tucker AS, Matthews KL, Sharpe PT. 1998. Transformation of tooth type induced by inhibition of BMP signaling. Science 282:1136-1138.
Tucker AS, Sharpe PT. 1999. Molecular genetics of tooth morphogenesis and patterning: the right shape in the right place. J Dent Res 78:826-834.
Tufro A, Teichman J, Woda C, Villegas G. 2008. Semaphorin3a inhibits ureteric bud branching morphogenesis. Mech Dev 125:558-568.
Tuisku F, Hildebrand C. 1994. Evidence for a neural influence on tooth germ generation in a polyphyodont species. Dev Biol 165:1-9.
Tummers M, Thesleff I. 2009. The importance of signal pathway modulation in all aspects of tooth development. J Exp Zool B Mol Dev Evol 312B:309-319.
Tummers M, Yamashiro T, Thesleff I. 2007. Modulation of Epithelial Cell Fate of the Root in vitro. Journal of Dental Research 86:1063-1067.

Ulupinar E, Datwani A, Behar O, Fujisawa H, Erzurumlu R. 1999. Role of semaphorin III in the developing rodent trigeminal system. Mol Cell Neurosci 13:281-292.
Unda FJ, Martin A, Hilario E, Begue-Kirn C, Ruch JV, Arechaga J. 2000. Dissection of the odontoblast differentiation process in vitro by a combination of FGF1, FGF2, and TGFbeta1. Dev Dyn 218:480-489.
Vaahtokari A, Vainio S, Thesleff I. 1991. Associations between transforming growth factor beta 1 RNA expression and epithelial-mesenchymal interactions during tooth morphogenesis. Development 113:985-994.
Vainio S, Karavanova I, Jowett A, Thesleff I. 1993. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell 75:45-58.
van den Boogaard MJ, Creton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, Cune M, Ploos van Amstel HK. 2012. Mutations in WNT10A are present in more than half of isolated hypodontia cases. J Med Genet 49:327-331.
van der Zwaag B, Hellemons AJ, Leenders WP, Burbach JP, Brunner HG, Padberg GW, Van Bokhoven H. 2002. PLEXIN-D1, a novel plexin family member, is expressed in vascular endothelium and the central nervous system during mouse embryogenesis. Dev Dyn 225:336-343.
Varela-Echavarria A, Guthrie S. 1997. Molecules making waves in axon guidance. Genes Dev 11:545-557.
Vastardis H, Karimbux N, Guthua SW, Seidman JG, Seidman CE. 1996. A human MSX1 homeodomain missense mutation causes selective tooth agenesis. Nat Genet 13:417-421.
Veerayutthwilai O, Luis NA, Crumpton RM, MacDonald GH, Byers MR. 2006. Peripherin- and CGRP-immunoreactive nerve fibers in rat molars have different locations and developmental timing. Arch Oral Biol 51:748-760.
Vilbig R, Cosmano J, Giger R, Rochlin MW. 2004. Distinct roles for Sema3A, Sema3F, and an unidentified trophic factor in controlling the advance of geniculate axons to gustatory lingual epithelium. J Neurocytol 33:591-606.
Vitriol EA, Zheng JQ. 2012. Growth cone travel in space and time: the cellular ensemble of cytoskeleton, adhesion, and membrane. Neuron 73:1068-1081.
Vizi S, Palfi A, Hatvani L, Gulya K. 2001. Methods for quantification of in situ hybridization signals obtained by film autoradiography and phosphorimaging applied for estimation of regional levels of calmodulin mRNA classes in the rat brain. Brain Res Brain Res Protoc 8:32-44.
Wada N, Maeda H, Hasegawa D, Gronthos S, Bartold PM, Menicanin D, Fujii S, Yoshida S, Tomokiyo A, Monnouchi S, Akamine A. 2014. Semaphorin 3A Induces Mesenchymal-Stem-Like Properties in Human Periodontal Ligament Cells. Stem Cells Dev.
Wang XP, Suomalainen M, Jorgez CJ, Matzuk MM, Werner S, Thesleff I. 2004. Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation. Dev Cell 7:719-730.
White FA, Behar O. 2000. The development and subsequent elimination of aberrant peripheral axon projections in Semaphorin3A null mutant mice. Dev Biol 225:79-86.

Whitlock JA, Richman JM. 2013. Biology of tooth replacement in amniotes. Int J Oral Sci 5:66-70.
Wilkinson DJ, Green J, Copp AJ, Cockroft DL. 1990. Postimplantation mammalian embryos: a practical approach. Oxford: Oxford University Press. xxi, 357 s. : ill. pp.
Williams-Hogarth LC, Puche AC, Torrey C, Cai X, Song I, Kolodkin AL, Shipley MT, Ronnett GV. 2000. Expression of semaphorins in developing and regenerating olfactory epithelium. J Comp Neurol 423:565-578.
Wong ML, Medrano JF. 2005. Real-time PCR for mRNA quantitation. Biotechniques 39:75-85.
Woodnutt DA, Wager-Miller J, O'Neill PC, Bothwell M, Byers MR. 2000. Neurotrophin receptors and nerve growth factor are differentially expressed in adjacent nonneuronal cells of normal and injured tooth pulp. Cell Tissue Res 299:225-236.
Worzfeld T, Puschel AW, Offermanns S, Kuner R. 2004. Plexin-B family members demonstrate non-redundant expression patterns in the developing mouse nervous system: an anatomical basis for morphogenetic effects of Sema4D during development. Eur J Neurosci 19:2622-2632.
Worzfeld T, Rauch P, Karram K, Trotter J, Kuner R, Offermanns S. 2009. Mice lacking Plexin-B3 display normal CNS morphology and behaviour. Molecular and Cellular Neuroscience 42:372-381.
Xiao T, Shoji W, Zhou W, Su F, Kuwada JY. 2003. Transmembrane sema4E guides branchiomotor axons to their targets in zebrafish. J Neurosci 23:4190-4198.
Yaron A, Huang PH, Cheng HJ, Tessier-Lavigne M. 2005. Differential requirement for Plexin-A3 and -A4 in mediating responses of sensory and sympathetic neurons to distinct class 3 Semaphorins. Neuron 45:513-523.
Yazdani U, Terman JR. 2006. The semaphorins. Genome Biol 7:211.
Yen AH, Sharpe PT. 2008. Stem cells and tooth tissue engineering. Cell Tissue Res 331:359-372.
Yokohama-Tamaki T, Ohshima H, Fujiwara N, Takada Y, Ichimori Y, Wakisaka S, Ohuchi H, Harada H. 2006. Cessation of Fgf10 signaling, resulting in a defective dental epithelial stem cell compartment, leads to the transition from crown to root formation. Development 133:1359-1366.
Yoshiba N, Yoshiba K, Nakamura H, Iwaku M, Ozawa H. 1996. Immunohistochemical localization of HLA-DR-positive cells in unerupted and erupted normal and carious human teeth. J Dent Res 75:1585-1589.
Yukawa K, Tanaka T, Bai T, Ueyama T, Owada-Makabe K, Tsubota Y, Maeda M, Suzuki K, Kikutani H, Kumanogoh A. 2005. Semaphorin 4A induces growth cone collapse of hippocampal neurons in a Rho/Rho-kinase-dependent manner. Int J Mol Med 16:115-118.
Yukawa K, Tanaka T, Yoshida K, Takeuchi N, Ito T, Takamatsu H, Kikutani H, Kumanogoh A. 2010. Sema4A induces cell morphological changes through B-type plexin-mediated signaling. Int J Mol Med 25:225-230.
Zhao H, Feng J, Seidel K, Shi S, Klein O, Sharpe P, Chai Y. 2014. Secretion of shh by a neurovascular bundle niche supports mesenchymal stem cell homeostasis in the adult mouse incisor. Cell Stem Cell 14:160-173.

Zhou Y, Gunput RA, Pasterkamp RJ. 2008. Semaphorin signaling: progress made and promises ahead. Trends Biochem Sci 33:161-170.