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Transmembrane Semaphorin 6A controls timing of entrance of the Sensory Innervation into mice mandibular first molar tooth germ

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Abstract

Background: Semaphorins are 9membrane-spanning and soluble proteins that have many important neuronal and non neuronal functions during development as well as in adult physiology. They regulate axon growth and guidance, angiogenesis, cell movement, and have functions in several organ systems. Semaphorin6A (sema6a) is a membrane-spanning protein, which acts as a repellant for sympathetic as well as sensory dorsal root axons. It has been reported that embryonic and postnatal murine dental mesenchyme expresses Sema6a mRNAs. Expression was found to be high early in development and then decreased in a temporal pattern that connected with new inhibitory/repulsion effects of dental mesenchyme observed in co-cultures.

Main objective: The aim of this study is to investigate the role of sema6a protein in tooth morphogenesis via exploring its role in regulation of timing of entrance of sensory nerve fibers into dental pulp of murine mandibular first molar tooth.

Methods: This is an *in vivo* study; immunohistochemistry was used to identify the location of an antigen in the tissue. Laboratory transgenic mice, antibodies, Optimum cutting tempreature (OCT) tissue tek embedding medium, Polymerase Chain Reaction (PCR) reagents, 3-amino 9-ethylcarbazole (AEC) chromogen, were the main materials used in the study. Microscopical methods enabled revealing the results.

Results: Serial sections of PostNatal day 0, 1, 3, 5 and 7 were examined under bright field microscope and revealed that sensory nerve fibers were prematurely present inside the dental pulp of sema6a knockout mice between PN0 and PN1. In wild type kind of mice sensory nerve fibers were absent at PN3 and numerous at PN5 and that was in accordance to previous studies which showed that they first enter between PN3 and PN4. Anti-peripherin antibody showed superior nerve detection over neurofilament 200.

Conclusions: Sema6a protein controlled the timing of entrance of sensory nerve fibers into the dental pulp of mandibular first molar tooth; hence it is a growth factor to be considered for oral tissue engineering.

Anti-peripherin antibody showed superior detection ability of nerve fibers over neurofilament 200. Pattern of penetration of nerve fibers was similar in both genotypes and this suggests other molecules are compensating sema6a absence.

Keywords: Semaphorins, Sema6a, sensory innervation, epithelial mesenchymal interaction, mandibular first molar tooth, transgenic mice.

Introduction

Tissue engineering and regenerative science is an innovative, widely trending field of science, understanding the molecular interaction controlling tooth development is essential in defining the elements needed for reconstruction of defects, using stem cells, and growth factors [1].

Establishment of tooth morphogenesis and nerve supply of the dental pulp is mediated by members of protein growth factor families[2]. Experimental gene expression studies as well as knockout mice have revealed some of these signaling pathways, e.g. Fibroblast growth factors (FGFs) and Bone morphogenic proteins (BMPs) and the semaphorin family [3].

Semaphorin is a large family of secreted and membrane linked proteins (Semaphorin Nomenclature Committee 1999), they have shown a variety of functional domains, together with their receptors they arbitrate repulsion as well as attraction actions including neuronal methods e.g. axon branching and fasciculation, as well as regulation of non neuronal processes e.g. cellular morphology[4], Cardiovascular system development [5] [6], angiogenesis [7] and tumor biology [8].

Semaphorin3a was found to be distinctively expressed in a regulated pattern around the mandibular first molar tooth germ [9], and it has shown that it controls timing of entrance of sensory nerve fibers into dental pulp of mandibular first molar in mice as nerve first entered at postnatal day 0 in sema3a deficient mice, while in wild type of mice they first enter at postnatal day 4 [10].

Semaphorin6a previously known as, semaq and semaVIa, is a membrane bound molecule that was first identified since it shared a structural comparability to sema1a [11]. Sema6a contains extracellular sema domain, transmembrane domain, and a cytoplasmic domain. It is found in humans, located at chromosome 5 and labeled as (SEMA6A), in rats/mice it is situated at chromosome 18 and marked as (sema6a), cattle, dogs and chimpanzee (SEMA6A) [12]. Sema6B/C and D all share similar structure with sema6a [13]. Sema6a mRNAs are expressed in various systems in mammals [14] [15]. Several studies have addressed the guidance role of sema6a protein in regulation of peripheral nervous system. The neuronal effect of sema6a in behavior alteration in mice has been investigated and results showed that absence of sema6a led to an interruption of cell organization, wiring and lamination in the limbic system and cortical neurons, which led to multiple variations in the electroencephalogram (EEG) when studied as well as hyper-exploratory behavior, changed social interaction, shortage in object identification, which are typical symptoms of psychosis [16].

Sema6A mRNAs were expressed in the mesenchyme around the tooth germ (i.e., target field of the dental innervation), this suggested that sema6a might have a role in regulation if dental innervation[17].

Murine mandibular first molar tooth germ has been used as an experimental model during embryonic and postnatal stages of tooth germ development to study molecular regulation of tooth formation [18]. Since steps of tooth development in mammals are similar. Morphology, histology, as well as the general features of sensory innervation of the pulp in different species e.g. rat, cat, monkey, and dog are similar to that in the human teeth [19].

In this study, to explore the role of sema6a protein in controlling the morphogenesis of sensory innervations of murine mandibular first molar we checked the timing of penetration of sensory nerve fibers into the dental papilla of mandibular first molar tooth of sema6a knockout mice and compared it to wild type mice., we stained serial sections of postnatal stages (PN0, 1, 3, 5 and 7) using

three layered immunohistochemistry via anti-peripherin antibody. We found that sensory nerve fibers first entered the dental papilla of mandibular first molar tooth of sema6a knockout mice between postnatal day 0 (PN0) and postnatal day 1 (PN1) which was earlier compared to wild type since pioneer sensory nerve fibers entered the dental papilla of mandibular first molar tooth in wild mice between postnatal day 3-4 which was a positive remarkable result confirming the role of sema6a protein in tooth morphogenesis.

Materials and Methods

Overview

This is an *in vivo* study; immunohistochemistry was used to identify the location of an antigen in the tissue [20]. Laboratory mice, antibodies, OCT tissue tek embedding medium, Polymerase Chain Reaction (PCR) reagents, AEC chromogen, were the main materials used in the study. Microscopical methods enabled revealing the results.

Animals

Laboratory mouse strains are widely used in *in vivo* craniofacial development investigations and studies of molecular mechanisms of mammalian teeth as they are easy to handle ear-mark and reproduce relatively fast since the pregnancy lasts for 19 days. Mice can also be genetically modified and their genome resembles humans [21].

Sema6a Knockout (deficient) strain is a transgenic mouse strain had been generated via gene modification/trapping methods [22], which allowed observation of sema6a *in vivo* functions [23]. The strain used in this study (C57BL/6, black fur) was generously offered to craniofacial development biology group by Dr. Kevin J. Mitchell, Smurfit Institute of Genetics and Institute of Neuroscience, Trinity College, Dublin, Ireland. Mice breeding colonies are kept in the animal facility department at Haukeland University Hospital, University of Bergen and fed with pellets and tap water.

Approval for the use of laboratory animals was obtained from the Department of Biomedicine. Faculty of medicine and dentistry, University of Bergen, all procedures were performed in accordance to Norwegian Animal Research Authority Guidelines.

Sema6a +/- mice were mated overnight and presence of vaginal plug marked embryogenesis and set as E0. Postnatal day 0 (PN0) marked the day mice were born, pups (mice newborns) from PN0, PN1, PN3, PN5 and PN7 age stages were collected.

Genotyping and IHC

The genotype analysis of sema6a protein was done by PCR as previously described by [24]. Following pups collection, heads of Post natal day 0,1 and 3 in addition to mandibles of PN5 and 7 were washed with phosphate buffered saline PBS and embedded in OCT Tissue Tek embedding medium.

Serial coronal 30 µm sections were cut using a cryostat, Post fixed in 4% paraformaldehyde PFA. Sections were Stained via three layered immunohistochemistry using rabbit anti-peripherin polyclonal antibody (Chemicon International, CA, USA) as described in [9],[10]. Serial sections were observed with high and low magnification objec-tives and representative bright and dark field images were taken with 5 0.15 NA and 10 0.3 NA objective using a Zeiss Axioskop 2 Plus microscope. Image plates were made with Adobe Photoshop CS4 software.

Results

Spatiotemporal localization of sensory nerve fibers during development of the dental papilla/pulp of mandibular first molar tooth

Using three layered immunohistochemistry via anti-peripherin antibody, serial sections of PN0, 1, 3, 5 and 7 of wild type (+/+) as well as sema6a deficient mice (-/-)

were stained and examined under bright field microscope, in both genotypes sections showed histological similarity nevertheless timing of entrance of sensory nerve fibers differed.

At PN0 the tooth germ is at the late bell stage, no enamel or dentine still formed, a mental nerve (thick double arrow in Figure 1, A1 and A2) located below the tooth germ and most importantly no sensory nerve fibers were observed inside the dental papilla of all mesial (20 sections), middle (following10 sections) and distal (20 final sections) in both wild type and sema6a-/- mutant mice.

PN1 was the first chronological stage in which there was a real difference in timing of penetration of sensory nerve fibers into the dental papilla of wild type+/+ and Semna6a mutant -/- mice. No sensory nerve fibers were detected inside the dental papilla in wild type tooth germ sections (Fig 1, B1) on the contemporary to the Sema6a-/- mice sensory nerve fibers were observed inside the dental pulp of the mandibular first molar tooth germ (Fig1, B2) , nerve fibers were thin long and short, heading towards a coronal direction.

At PN3 histological addition is the beginning of amelogenesis and dentinogenesis had begun and a thin layer of enamel and dentine could be observed in both comparable mice types wild type +/+ (Fig 1, C1) and Sema6a knock out -/- (Fig 1, C2). Sema6a-/- sections showed more branching sensory nerve fibers inside its dental pulp. There were still no sensory nerve fibers the dental pulp of wild type mice.

Postnatal Day 5 was a chronological age stage where wild type mice showed sensory nerve fibers inside their dental pulp (Fig 2, A1). Sema6a mutant-/- mice also showed nerve fibers inside the dental pulp (Fig 2, A2). In PN7 findings were similar in wild type and sema6a mutant mice (Fig 2, B1 and B2) and with more numerous sensory nerve fibers inside the dental pulp of wild type and Sema6a-/- mice, histological morphology showed thicker enamel and dentine layers in both genotypes.

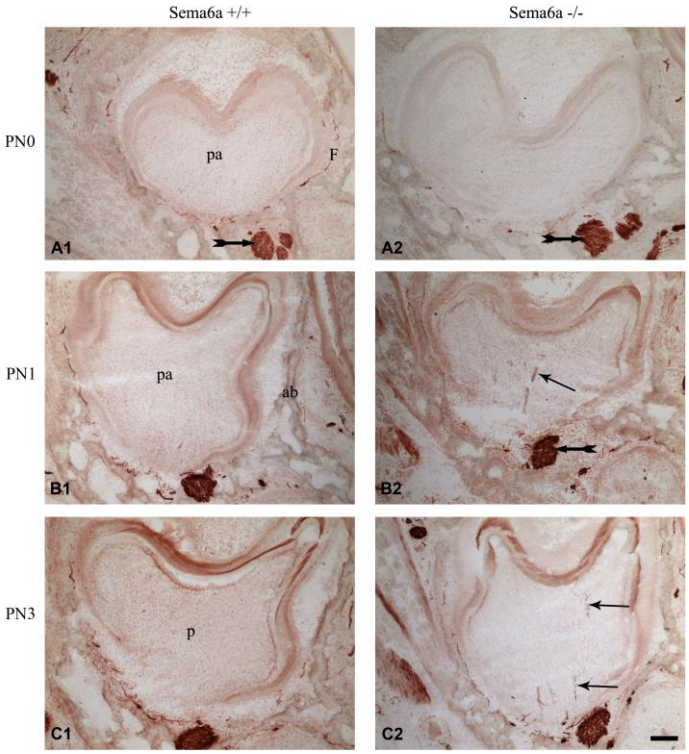

Figure 1. Three layered Immunohistochemistry performed with anti-peripherin antibody on 30 um sections of mandibular first molar tooth of wild type and sema6a -/- mice.

At PN0 no nerve fibers were seen inside the dental papilla of mandibular molar tooth of wild type (A1) and sema6a-/- (A2). The thick double arrow points at the mental nerve, which was present at all sections of the lower jaw. At PN1 no nerve fibers were seen inside the mandibular molar tooth of wild type mice (B1). Thin, long nerve fibers were detected inside the dental papilla of mandibular molar tooth of sema6a -/- (B2). At PN3 there were still no nerve fibers inside the dental pulp of wild type mice (C1) and numerous nerve fibers were present inside the dental papilla of sema6a mutant -/- mice (C2). Thin black arrow= sensory nerve fibers. Thick double arrow= mental nerve. pa= papilla, F= follicle, ab=alveolar bone, p= pulp, Scale car =100 um.

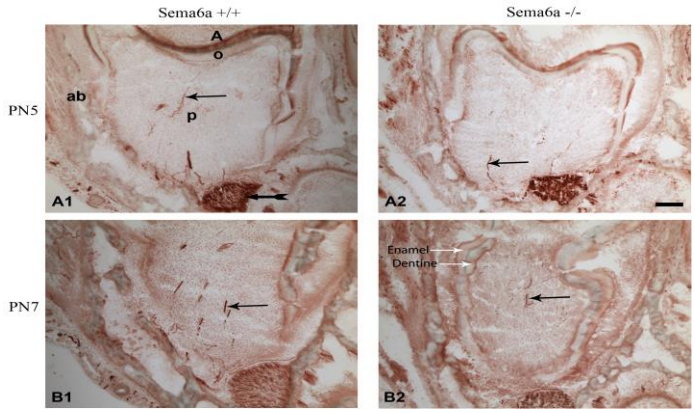

Figure 2 Three layered immunohistochemistry performed with anti-peripherin antibody on 30 um sections of mandibular first molar tooth of wild type and sema6a -/- mice. (A1 and A2 =PN5, B1 and B2= PN7. Thin plane arrow at A1 shows sensory nerve fibers inside the dental pulp of PN5 sema6a +/+ mice, Thick double arrow shows the mental nerve, which was always present in all sections. White arrows at B2 show the enamel and dentine layers at PN7 sema6a -/- mice). A= ameloblast, O=odontoblast, ab= alveolar bone, Scale bar = 100 um.

Another interesting finding was that number of detected nerve fibers in the dental papilla/pulp was different with anti-peripherin than anti-neurofilament 200 antibody using double immunofluorescence method. Anti-peripherin antibody showed superior detection ability of nerve fibers over neurofilament 200.

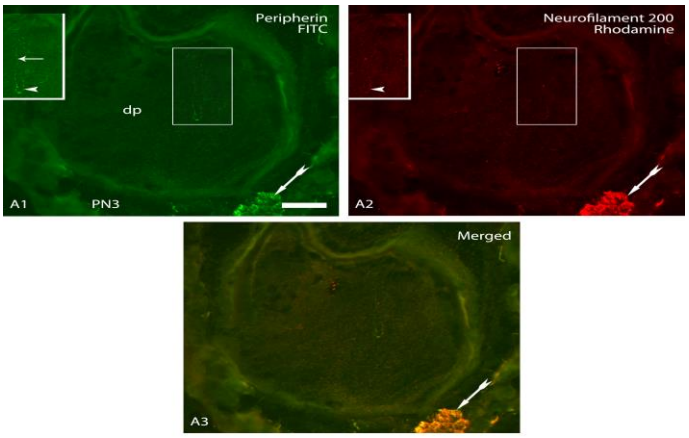

Figure 3 Double Immunofluorescence of 30 um sections of mandibular first molar tooth germ.

Chronological age is postnatal day3 (PN3) stained with anti-peripherin antibody and anti-neurofilament 200, showing positive immunoreaction to anti-peripherin and anti-neurofilament 200 antibodies and a combined image. Thin white arrow and the arrow head inside the zoomed rectangle in A1, shows sensory nerve fibers detected by anti-peripherin antibody, arrow head in the zoomed rectangle in A2 shows that same area stained with anti-neurofilament 200 but with less detected nerve fibers. Double thick arrow shows the mental nerve. dp= dental pulp. Scale bar = 100 µm

Discussion

Rationale for the study

Sensory nerve fibers are guided to their final target organs with the help of axon guidance molecules and their receptors. Such molecules belong to protein families including semaphorins, slits, netrins and ephrins [25]. Timing and pattern of penetration of dental pulp nerve fibers is firmly spatio-temporally regulated by repulsive and attractive molecules [26] [27].

The main hypothesis of this study states that sema6a protein acts as a local axon repellant that regulates timing and penetration pattern of sensory nerve fibers into the mandibular first molar tooth in mice.

Two findings based the rationale for conducting this study, i) Sema3a transcripts were found to be expressed in areas around the tooth germ of mandibular first molar where axons avoided entrance [26][27], ii) Sema6a protein mRNA transcripts were found to be expressed in the mandibular dental mesenchyme [28]. An *in vitro* study was conducted by Lillesaar and Fried 2004; the aim of the study was to investigate the ability of dental mesenchyme to repel explants from trigeminal ganglion and explore if semaphorins were involved in this action *in vitro*. They co-cultured murine embryonic and postnatal mandibular and trigeminal ganglion (TG) tissues in collagen and using

PCR found out that some members of semaphorin family including sema3a/c/f, sema4f, sema5b, sema6b/c and most importantly sema6a transcripts were expressed in dental papilla. In Embryonic day 11 (E11) expression was present but it reached the highest level at embryonic day 13-15. At the same time-line (E13-15), it has been found that trigeminal ganglion axons were repelled. The expression of sema6a transcripts decreased with increasing age, and this was also in accordance to the fact that attraction of trigeminal axons took place later at postnatal stages. This suggests that sema6a protein might have a role in dental axon guidance [28]. Another recent study found out that sema3a controls the timing of entrance of sensory nerve fibers into the dental pulp of mandibular incisor tooth [17].

The second rational point for conducting our study is the fact that the cellular expression of sema3a mRNA transcripts in areas that port the mesenchymal dental axon pathway around the developing tooth germ of mandibular first molar tooth in mice, was related to its role in controlling time of entrance of sensory nerve fibers into the dental papilla/pulp of mandibular first molar tooth germ. Sema3a was first identified at E11.5 and this was at the dental mesenchyme between thickened dental epithelium and the buccal nerve, at E12.5 sema3a was found at the mesenchyme next to the molar nerve, which is a branch of the trigeminal nerve, and later in the same bud stage sema3a was found to be in the mesenchyme under which the molar nerve divided into buccal and lingual branches. At postnatal day1 sema3a transcripts were expressed in the area of the prospect pulp floor and in the periphery of the pulp, adjacent to epithelial cervical loops of the first molar tooth. At postnatal day 4 Sema3a transcripts were expressed in preodontoblasts adjacent to the epithelial cells of the pulp floor as well as next to the epithelial root sheaths, which form the mesial and distal roots of the molar. The areas in the mesenchyme, which lack sema3a, defined the sites of the secondary apical foramina through which the sensory nerve fibers entered the dental pulp [26].

Conclusions

Sema6a protein does control the timing of entrance of sensory nerve fibers into the dental papilla of mandibular first molar tooth germ; hence it is a growth factor to be considered for oral tissue engineering.

Anti-peripherin antibody showed superior detection ability of nerve fibers over neurofilament 200

Double knock out studies are needed to explore the effect of presence of more than one semaphorin family member on the tooth morphogenesis.

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