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Semaphorin 3A controls timing and patterning of the dental pulp innervation

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ABSTRACT

Timing and patterning of dental pulp innervation are strictly spatio-temporally regulated but it is still not known how they are controlled at molecular level. We analyzed postnatal innervation of the dental pulp in the mandibular first molar of mice deficient for Semaphorin 3A (Sema3A) axon repellant molecule. Immunohistochemical localization of nerve fibers on serial sections covering the whole tooth germs using anti-peripherin antibody revealed that nerve fibers were prematurely present within the preodontoblast layer next to the inner enamel epithelium already at PN0 in *Sema3A*$^{-/-}$ mice. In contrast, in the wild-type (*Sema3A*$^{+/+}$) mice nerve fibers were seen in the pulp only after enamel formation at PN3. The nerves in *Sema3A*$^{-/-}$ pulp were notably defasciculated and thinner compared to that of *Sema3A*$^{+/+}$ mice. A premature formation of an abnormal, enlarged nerve plexus with a high number of arborizations was apparent in the pulp–dentin border target area in *Sema3A*$^{-/-}$ already at PN2 whereas in the wild-type mice the first sign of plexus formation was seen at PN7. The expression of mRNAs for Ngf, Gdnf and Ncam neuroregulatory molecules in mandibular molar as well as receptors for neurotrophic factors and class 3 semaphorins including Sema3A (TrkA, p75, TrkB, TrkC, Ret, Npn1, Npn2, PlxA4) in trigeminal ganglia were not altered in the *Sema3A*$^{-/-}$ mice. Collectively, this data show that Sema3A serves an essential role in molar tooth pulp innervation controlling the timing of nerve fiber penetration into the pulp, their patterning and the formation of nerve plexus at pulp–dentin border area, and provide further support for the hypothesis that tooth innervation is regulated by the coordinated activity of locally expressed neuroregulatory molecules exerting positive and negative influences on growing dental nerve fibers.

1. Introduction

The tooth, in particular the two-rooted mandibular first molar, is an advantageous, well-characterized model system for studying molecular mechanisms of development of peripheral innervation (Fried et al., 2007; Luukko et al., 2005,2008). The dental pulp is an important target tissue for the sensory and sympathetic nerves, which originate from the trigeminal and superior cervical ganglion, respectively. Sensory axon navigation to the developing tooth takes place in a highly spatio-temporally controlled manner and is tightly linked with advancing crown morphogenesis and tooth-specific cell differentiation (Luukko et al., 2005,2008).

In mouse, the first trigeminal axons reach the mandibular first molar tooth germ at the embryonic bud stage (Kettunen et al., 2005,2007; Loes et al., 2002; Mohamed and Atkinson, 1983), but the innervation of the dental pulp does not commence before the final shape of the crown which is largely established and the enamel and dentin formations has started at around postnatal day 3–4 (PN3–4) (Moe et al., 2008; Mohamed and Atkinson, 1983). Sympathetic nerves penetrate the mouse molar pulp after the onset of root formation, at around PN9 (Moe et al., 2008). Trigeminal nerve fibers penetrate the mandibular molar pulp specifically through the presumptive sites of the mesial and distal roots, and subsequently innervate the pulp–dentin border target area (Luukko et al., 2008), consisting of subodontoblastic region where they form a dense network of fibers adjacent to the roof of the pulp chamber, the odontoblast layer, and predentin (Byers, 1984). However, it is still not known how the timing and patterning of molar dental pulp innervation are controlled.

Some light has been shed on the regulatory mechanisms controlling the establishment of the tooth innervation. The tooth

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germ regulates the formation of its own nerve supply by exerting developmentally changing influence on growing nerve fibers (Erdelyi et al., 1987; Lillesaar and Fried, 2004; Luukko et al., 1996,1997a,1997b). There is increasing evidence suggesting that locally produced secretory and membrane-bound neuroregulatory signaling molecules of different families control tooth innervation (Fried et al., 2007; Luukko et al., 2005,2008; Magloire et al., 2010). One of the key regulatory molecules is Semaphorin 3A (Sema3A), a secreted axon repellant protein, the inactivation of which leads to defasciculation and patterning defects in peripheral nerves in mouse embryos (Taniguchi et al., 1997). In the embryonic tooth germ, Sema3A shows distinct, developmentally regulated expression patterns in the mesenchymal exclusion areas avoided by growing dental axons. These areas become ectopically innervated in Sema3A-deficient mice demonstrating that Sema3A controls axon navigation and patterning of the embryonic mouse molar tooth germ (Kettunen et al., 2005).

The distinct cellular expression domains of *Sema3A* at the middle part of the base of the postnatal molar pulp and around the presumptive areas of the mesial and distal roots prior to and during the pioneer nerve ingrowth (Kettunen et al., 2005) suggest an important role for Sema3A in regulation of dental pulp innervation. To investigate this we analyzed the innervation of the mandibular first molars of Sema3A-deficient (*Sema3A*$^{-/-}$, *Sema3A*$^{+/-}$) and wild-type (*Sema3A*$^{+/+}$) mice between postnatal days 0 and 7 (PN0–7). Moreover, in order not to miss any nerve fibers and to obtain information regarding pattern of nerve fibers ingrowth into dental pulp, immunohistochemical analysis was performed on serially sectioned tooth germs.

2. Materials and methods

2.1. Animals

The animal use was approved by Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen under the surveillance of Norwegian Animal Research authority. Two different lines of Sema3A-deficient mice on C57BL/6 and CD1 backgrounds described earlier were analyzed (Taniguchi et al., 1997). *Sema3A*$^{+/-}$ mice were mated overnight and the day of birth was designated as postnatal day 0 (PN0). Pups from PN0, PN1, PN2, PN3, PN5 and PN7 stages were collected. Genotyping of the mice was performed as described previously (Taniguchi et al., 1997). Tissues were embedded in Tissue-Tek OCT (Sakura Finetek Europe B.V., The Netherlands) (for immunohistochemistry and immunofluorescence) and paraffin (for in situ hybridization). For the localization of nerve fibers we compared *Sema3A*$^{+/+}$, *Sema3A*$^{+/-}$ and *Sema3A*$^{-/-}$ mice from the same litter. At least two sets of serial frontal frozen sections of first molars per genotype per age were examined. Post fixation was done with 4% paraformaldehyde.

2.2. Double confocal immunofluorescence

In order to address the ability of peripherin antibody to detect trigeminal dental nerves we compared the expression patterns of rabbit polyclonal anti-peripherin antibody (AB1530, Chemicon international, CA, USA) (1:250 dilution) with mouse monoclonal antibody against heavy chain neurofilament called NF200 (N 0142, Sigma-Aldrich, Inc, USA) (1:250 dilution) using double fluorescence immunohistochemistry in PN3 mandibular first molar (30 μm thick section). We used FITC goat anti rabbit (for peripherin) (1:500 dilution) and rhodamine donkey anti mouse (for NF200) (1:500 dilution) as secondary antibodies. Imaging was done with Leica SP5 AOBS confocal microscope using 488 nm Argon ion and 561 nm DPSS lasers. Sequential scanning method was used to minimize crosstalk and bleed through problems. In negative control sections where one primary antibody was omitted showed no fluorescence in the corresponding channel (data not shown). Optical sections were volume rendered with maximum intensity projection method using the Imaris software (Bitplane, Switzerland). Snapshots of volume rendered images were used to make image plate with Adobe Photoshop CS4 software (Adobe Systems Incorporated, CA, USA).

2.3. Immunohistochemistry

Immunohistochemistry on fresh frozen sections (30 μm thick) was carried out as described using rabbit polyclonal anti-peripherin antibody (1:250 dilution) (AB1530, Chemicon international, CA, USA) (Kettunen et al., 2005; Loes et al., 2002; Moe et al., 2008). Avidin biotin peroxidase complex method (PK-6100, VECTASTAIN® Elite ABC Kit, Vector Laboratories, USA) was performed according to the instructions using 3-amino-9-ethylcarbazole (AEC) (A6926, Sigma-Aldrich, Inc, USA) as a chromogen. No specific immunoreactivity was observed in the negative control sections. All sections were observed under Zeiss Axioskop 2 Plus microscope and images were taken using Spot Insight digital camera (Diagnostic Instruments Inc. Sterling Heights, MI). The image plate was made using Adobe Photoshop CS4 software.

2.4. Thick tissue confocal imaging

In order to trace the course of nerve fibers as much as possible, we used thick section (100 μm thick) immunofluorescence method. PN7 mandibles from *Sema3A*$^{-/-}$ and *Sema3A*$^{+/+}$ mice were cut with cryotome and stained with rabbit polyclonal anti-peripherin antibody (AB1530, Chemicon international, CA, USA) (1:250 dilution) using floating section method. Secondary antibody was Cy3 donkey anti rabbit antibody (1:250 dilution). Tissue sections were cleared with BABB (benzyl alcohol/benzyl benzoate) solution and placed on the cavity slides using the BABB solution as the mounting medium. Imaging was done with Leica SP5 AOBS confocal microscope using 561 nm DPSS laser. Optical sections were volume rendered with maximum intensity projection method using a Imaris software (Bitplane, Switzerland). Snapshots of volume rendered images were used to make image plate with Adobe Photoshop CS4 software. Volume rendered images were also animated using Imaris software (Bitplane, Switzerland) and further processed with Quicktime Pro software (Apple Inc, USA).

2.5. In situ hybridization

For sectional in situ hybridization, mouse *Ngf*, *Gdnf*, *Ncam*, *TrkA*, *p75*, *Ret*, *Npn1*, *PlxA4*, *Npn2*, *TrkB* and *TrkC* cDNAs were used for in vitro transcription of ^{35}S-UTP- antisense and sense probes (Kettunen et al., 2005; Luukko et al., 1996,1997a,1997b; Sijaona et al., 2012). In situ hybridization was performed as described (Kettunen and Thesleff, 1998; Luukko et al., 1996). No specific hybridization signal was detected in sections hybridized with control sense probes (not shown).

3. Results

3.1. Localization of nerve fibers in the postnatal Sema3A-deficient molars

Peripherin has been used to detect dental nerve fibers in the developing tooth (Kettunen et al., 2005; Loes et al., 2002; Luukko,