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REGULAR ARTICLE

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Expression patterns of neurotrophic factor mRNAs in developing human teeth

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Abstract Neurotrophic factors regulate survival, differentiation, growth and plasticity in the nervous system. In addition, based on their specific and shifting temporospatial expression patterns, neurotrophic factors have been implicated in morphogenetic events during tooth development in rodents. To determine whether these findings in rodents could be related to humans, we have now studied nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), glial cell-line derived neurotrophic factor (GDNF), and neurturin (NTN) mRNA expression patterns in developing human teeth during gestational weeks 6.5–11. Using in situ hybridization histochemistry, we found distinct and specific patterns of neurotrophin and GDNF mRNA expression in the developing human teeth. NGF mRNA labeling was weak and confined predominantly to the dental papilla. BDNF mRNA labeling was stronger than NGF mRNA and was seen in the mesenchyme located lateral to the dental organ, as well as in epithelial structures (inner dental epithelium and enamel knot). NT-3 mRNA was observed in the dental papilla and in the area of the cervical loop. NT-4 mRNA was expressed in both oral and dental epithelia in all stages studied. GDNF mRNA was found in the dental follicle and at different sites in the inner dental epithelium. Weak NTN mRNA labeling was also found in the developing teeth. Based on these findings, we suggest that neurotrophins, GDNF and NTN might be involved in morphogenetic events during early stages of tooth development in humans. Protein gene product (PGP) 9.5-immunoreactive nerve fibers were observed in the dental follicle by 11 weeks coinciding with the labeling for neurotrophic factor mRNAs in this structure. This suggests that these neurotrophic factors might be involved in the innervation of dental structures. The rich expression of neurotrophic factors in developing dental tissues suggests that developing, or possibly adult, dental tissue might be used as an allograft source of trophic support for diseases of the nervous system.

Keywords Neurotrophin · Development · Tooth differentiation · Odontogenesis · Epithelial-mesenchymal interactions · Human

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Introduction

Tooth development is initiated through a complex series of successive and reciprocal interactions between the oral epithelium and the underlying ectomesenchymal cells. Oral epithelium provides the initial signals for neural crest-derived ectomesenchyme, both tissues then reciprocally participating in tooth formation (e.g., Lumsden 1987; Kollar and Mina 1991; Slavkin 1991). The mesenchyme, in turn, induces development of the enamel organ (Mina and Kollar 1987; Lumsden 1988). The interactions involve growth factors, transcription factors and their receptors as well as extracellular matrix components (Heine et al. 1987; Ruch 1987; MacKenzie et al. 1991; Orr-Urtreger et al. 1991; Satokata and Maas 1994; van Genderen et al. 1994; Matzuk et al. 1995a, 1995b; see also Thesleff et al. 1995). Teeth are richly innervated and the developing mammalian tooth becomes innervated when root formation commences and during the subsequent tooth eruption (Fristad et al. 1994). Subodontoblastic, odontoblastic and predentinal axonal networks are formed and axons are present in cuspal dentinal canals by the time teeth have become functional (Byers 1980). Interestingly, teeth in the osteopetrotic mutant mice become innervated in spite of the presence

of bone abnormalities and failure of root formation (Nagahama et al. 1998). This indicates that dental pulp innervation might be regulated by local pulpal events and interactions between the developing dental pulp and the ingrowing trigeminal nerve fibers. One way of explaining the events associated with dental pulp innervation has been based on expression of potent molecules, such as neurotrophic factors, in the dental pulp that initiate and orchestrate these events.

Neurotrophic factors play an important role in regulating neuronal survival and differentiation (see Lewin and Barde 1996). Earlier work from our laboratory (Nosrat et al. 1996b, 1997d, 1998) and others (Luukko et al. 1996, 1997a, 1997b) have shown that the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), as well as glial cell-line derived neurotrophic factor (GDNF) and the corresponding receptor complex mRNAs, are present in the developing tooth organ, as well as in trigeminal neurons innervating the teeth. It has been shown that NGF is required for differentiation of cranial neural crest cells into tooth organs by in vitro organ culture studies (Amano et al. 1999). At the same time, tooth innervation is severely reduced or lost in NGF or TrkA knockout mice, respectively (Byers et al. 1997; Matsuo et al. 2001), and anti-NGF treatment of neonatal rat pups reduces the amount of sensory axons in the dental pulp (Qian and Naftel 1996). NGF is also upregulated after experimental tooth injury (Byers et al. 1992; see also Woodnutt et al. 2000). These studies indeed indicate multiple roles for NGF in teeth. GDNF has also been proposed to be involved in the regulation of innervation of teeth postnatally (Luukko et al. 1997b; Nosrat et al. 1998).

However, some of the molecules that influence the ingrowing nerve fibers are also expressed at early stages of tooth initiation and development in rodents (Loes et al. 2001a, 2001b; Nosrat et al. 1998; Luukko 1998; see also Fried et al. 2000), suggesting not only that they are involved in tooth innervation, but that they are also involved in tissue interactions that lead to formation and morphogenesis of the teeth. To further elucidate the possible roles of neurotrophic factors in this process in humans, we used in situ hybridization to determine the specific sites of neurotrophin gene activity in human teeth. We found specific expression patterns for neurotrophin and GDNF mRNAs during tooth development.

Materials and methods

Tissue pieces were obtained following elective abortions (gestational weeks 6.5–11). The age was mainly estimated by evaluating several anatomical features such as morphology of organs, including the appearance and maturity of the central nervous system, extremities, and start of ossification in certain bones. Procedures were approved by the Human Ethics Committee of the Karolinska Institute, Stockholm, Sweden. Serial sections (14 μm) of the fetal tissues were cut on a cryostat and thaw mounted onto coated slides (ProbeOn, Fisher).

In situ hybridization histochemistry

Non-overlapping 50-mer oligonucleotide probes complementary to human NGF (GenBank accession number V01511, starting of bases 2149 and 2245, Ullrich et al. 1983), human BDNF (GenBank accession number M37762, starting of bases 333 and 419, Jones and Reichardt 1990), pig BDNF (GenBank accession number X16713, bases 250–298, Leibrock et al. 1989), human NT-3 (GenBank accession number M37762, starting of bases 225 and 740, Jones and Reichardt 1990), and human GDNF (GenBank accession number L19063, starting of bases 256 and 340, Lin et al. 1993) were synthesized (DNA technology, Aarhus, Denmark) and 3'-end labeled with ^{35}S-dATP. A 50-mer random control probe was also used (Scandinavian Gene Synthesis, Nosrat and Olson 1995). Procedures for in situ hybridization were as described by Dagerlind et al. (1992); see also Nosrat et al. (1996a). To increase the signals for the studied neurotrophic factors, the two non-overlapping human probes were combined after the specificity of the probes had been characterized. Sections were hybridized with specific and control probes for 16 h at 42°C. Slides were dipped in photographic emulsion (Kodak NTB2, diluted 1:1 in distilled water) and exposed for 8 weeks. After developing the slides were counterstained with cresyl violet and mounted.

Photomicrographs of the sections were scanned and, unless otherwise stated in the figure legends, digitally processed as for photoprints regarding brightness and contrast.

Positive control procedures included: (1) use of two different non-overlapping probes for a given mRNA species in our studies, (2) observation of correct labeling patterns in known areas (e.g., brain), and (3) microscopy carried out by two independent observers. Negative controls included: (1) the inclusion of a random control probe, (2) the fact that specific probes failed to label irrelevant structures and (3) the probes functioning as controls to each other since they had similar GC contents.

The specificity of the in situ hybridization procedure is dependent on high stringency conditions (in particular rinsing temperatures) and the positive and negative controls mentioned above. Detection of positive autoradiographic signals was based on serial observation of accumulation of silver grains in the emulsion above specific cells and tissues identified by the staining procedures. Only cells and areas over which silver grain accumulations were clearly above the surrounding background level, detectable by dark-field microscopy and a primary magnification of ×10 or less, were regarded as positive. To allow direct comparisons of distribution of different species, serial sectioning and labeling of consecutive sections with different probes were used. All comparisons were thus based on tissues sectioned, hybridized, exposed to emulsion, and further processed together. Processing several such pairs gives adequate information and is clearly superior to individual sectioning using one probe at a time.

Immunohistochemistry

To localize nerve fibers in the developing teeth and related structures, fetal tissue was immersion fixed in formalin-picric acid mixture (4% paraformaldehyde, 0.4% picric acid) in 0.1 M phosphate buffer (pH 7.2–7.4) overnight, rinsed in 10% sucrose solution containing 0.01% sodium azide (Merck, Germany) several times, and then dipped in OCT compound (Tissue-Tek, Miles Inc., USA) and frozen on dry ice. Fourteen-micrometer-thick sections were collected on chrome alum gelatin-coated slides and processed according to the indirect immunofluorescence technique (Hökfelt et al. 1973). Sections were rinsed (3×10 min) in 0.1 M phosphate-buffered saline (PBS) (1 l PBS; 0.1 M $Na_2HPO_4 \times 2H_20$ and $NaH_2PO_4 \times H_20$, pH 7.4, 4 g NaCl, 0.2 g KCl) and incubated for 24–48 h in a humid chamber at 4°C in antisera against protein gene product 9.5 (PGP, diluted 1:400; Biogenesis Ltd., Great Britain) in 0.1% PBS containing 0.3% Triton X-100. After rinsing in PBS (3×10 min), sections were incubated with appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC) diluted in 0.1% PBS in 0.3% Triton X-100 in a humidified cham-

Table 1 Comparison of patterns of expression of neurotrophic factors in developing human teeth (*ND* not detected, below detection level)

	6, 5–8 weeks	9 weeks	10 weeks	11 weeks
NGF	Strong labeling in the mesenchyme beneath the oral epithelium of the upper and lower jaws	Moderate labeling in the dental papillae	Weak scattered labeling in the dental papillae and dental follicle	ND
BDNF	Labeling in the mesenchyme of the upper and lower jaws, in the mesenchyme located lateral to the tooth anlagen, subepithelially in the anterior parts of the mandible	As in prior stages, additional mesenchyme located lateral to mesenchyme located lateral to the tooth anlagen, dental lamina, lateral sides of the dental follicle, medial and lateral parts of the inner dental epithelium, in the enamel knot, in the stellate reticulum in the area of the cervical loop, in the cervical loop, and in the skin epithelium	Labeling in the subepithelial labeling detected in the mesenchyme, in the mesenchyme lateral to the tooth anlagen, dental lamina, dental follicle, inner dental epithelium, enamel knot, and cervical loop area	Labeling in the mesenchyme lateral to the tooth anlagen, dental lamina, the entire dental follicle, and inner dental epithelium
NT-3	Broad labeling in the anterior part of the upper and lower jaws, in the oral epithelium and in the subepithelial mesenchyme. Moderate to strong labeling in the dental papillae	As in prior stages, additional labeling detected in the mesenchyme lateral to the tooth anlagen, dental follicle, and the basal layer of the dental lamina Strong labeling in the dental papillae	Labeling in the subepithelial mesenchyme, dental papillae (weaker than at 9 weeks), in the anterior parts of the oral epithelium, in the mesenchyme lateral to the developing teeth, and in the enamel knot and cervical loop	Labeling in the mesenchyme lateral to the developing teeth (weaker than at 10 weeks), dental papillae (weaker than at 10 weeks), and cervical loop (weaker than at 10 weeks)
NT-4	Strong labeling in the epithelium, dental lamina, and in the inner and outer oral dental epithelia (i.e., in epithelially derived tissue compartments)	As previous stages, but labeling appeared stronger in stellate reticulum	Similar to previous stages	Similar to previous stages but additional labeling was also found in the enamel knot
GDNF	Labeling in the differentiating dental papillae and dental follicle medially	Subepithelial labeling in anterior parts of the oral cavity, medial and lateral sides of the inner dental epithelium, and in the dental follicle	As at 9 weeks as well as in the enamel knots	As at 10 weeks
NTN	Labeling in the oral epithelium, and lateral part of the dental lamina	Subepithelial labeling in anterior parts of the oral cavity, inner dental epithelium, and cervical loop	General weak labeling in structures as mentioned for previous stages	ND

ber for 1.5 h at room temperature in darkness. Following a final rinse in PBS (3×10 min), sections were mounted in 90% glycerol in PBS containing 0.1% phenylenediamine. Photomicrographs were processed as above.

Results

In situ hybridization histochemistry

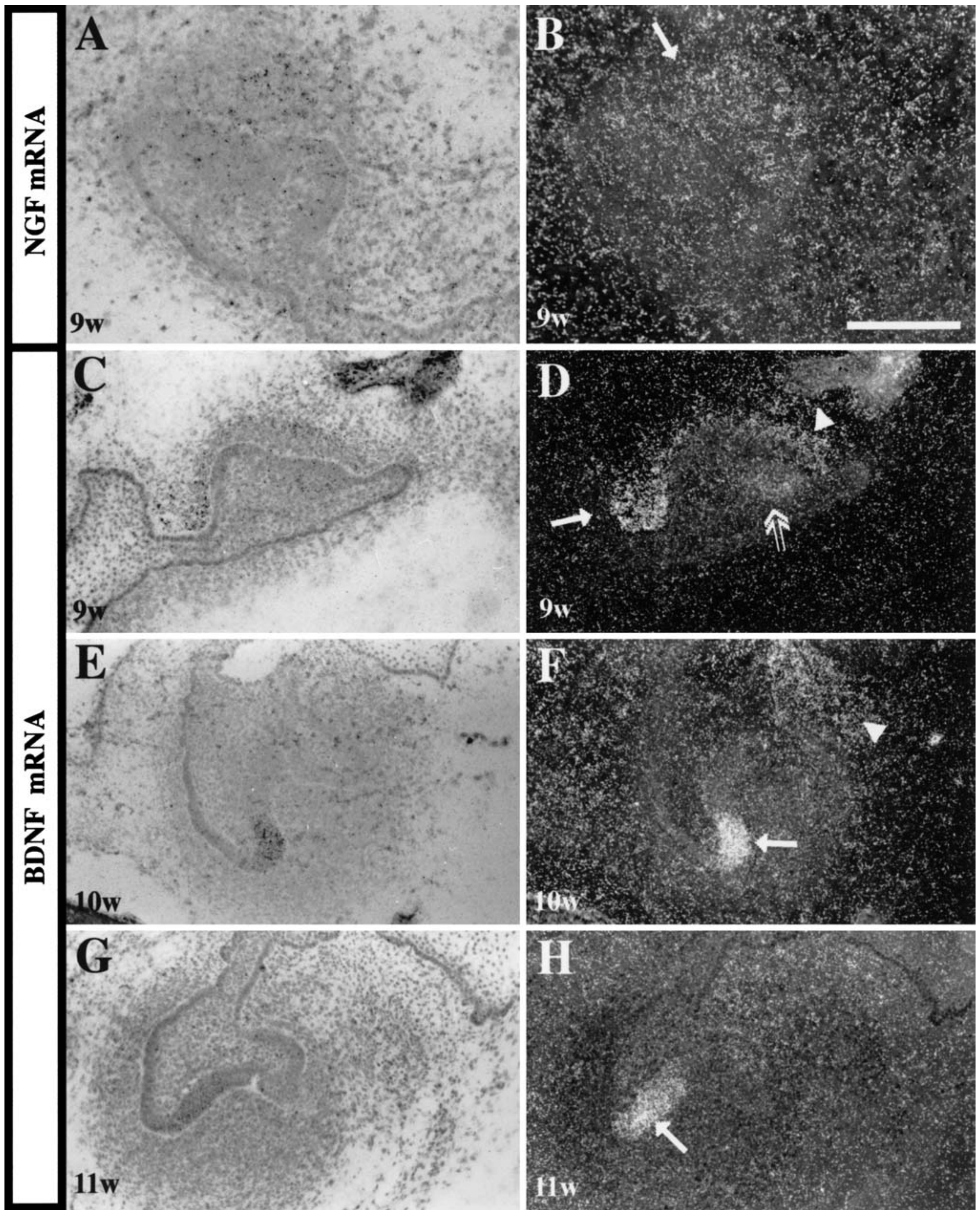
We describe the presence of different mRNA species in relevant tissues and structures in proximity to the developing teeth, followed by a detailed description of labeling in developing teeth. Whenever a difference was noticed in the presence of different mRNA species among different teeth, we divided the teeth into anterior and posterior teeth, denoting incisors or canines and deciduous molar teeth. A summary of the findings is given in Table 1.

NGF mRNA

At 6.5 weeks of gestation, strong labeling of the mesenchyme underneath the oral epithelium was detected. At 9 weeks, labeling was found in the dental papillae (9 weeks, Fig. 1A, B) of the developing teeth. At 10 weeks, in addition to the labeling in the dental papillae, labeled patched areas were observed in the dental follicle. At 11 weeks of gestation, NGF levels were below detection.

BDNF mRNA

At 6.5 weeks of gestation, a broad labeling was observed in the mesenchyme of the upper and lower jaws. At 8.5 weeks, strong labeling was detected in the mesenchyme lateral to the developing teeth. Strong labeling was also observed in the inner dental epithelium on the medial side and the fungiform papillae of the tongue


NGF mRNA
BDNF mRNA
A
B
C
D
E
F
G
H
9w
9w
9w
9w
10w
10w
11w
11w

(positive control). At 9 weeks, broad labeling was observed in the developing maxillary and mandibular processes. Labeling was seen in the skin epithelium but not in the oral epithelium. Interestingly, BDNF mRNA labeling was also found in the subepithelial mesenchyme in both upper and lower jaws. Generally, strong labeling was observed lateral to the tooth anlage (i.e., lateral to the outer dental epithelium in both upper and lower jaw teeth) as well as lateral to the dental lamina (Fig. 1C, D). This mesenchymal labeling would start at the vestibular lamina, and extend laterally in the lateral side of the dental follicle to where it covered the dental papillae. The labeling, however, did not extend to the medial side of the dental follicle. Labeling was also observed in lateral parts of the outer dental epithelium extending to the cervical loop. Occasionally, whenever labeling was seen in the lateral outer dental epithelium, it was also seen in medial aspects of the inner dental epithelium extending to the cervical loop. The labeling in the medial side of the inner dental epithelium was, however, stronger than the labeling in the lateral side of the outer dental epithelium. Labeling was also seen in the area of the enamel knot. Weak labeling was found in the stellate reticulum in the transitional areas between outer and inner dental epithelia in the area of the cervical loop. Labeling was also observed in the epithelial cells of the cervical loop area.

At 10 weeks of gestation (Fig. 1E, F), the labeling generally resembled that of 9 weeks except for the labeling in the mesenchyme lateral to the dental lamina, which was observed less frequently. Labeling in the medial side of the inner dental epithelium and in the dental follicle was stronger at 10 weeks compared to 9 weeks. BDNF mRNA labeling had increased both in intensity and extent in the medial parts of the inner dental epithelium. Interestingly, labeling was also seen in the area of the enamel knot. Labeling in the lateral side of the outer dental epithelium was not observed at 10 weeks. A distinct labeling was seen in the cervical loop on the lateral sides of developing teeth, resembling a shift of the labeling from the lateral sides of the outer dental epithelium towards the cervical loop area. Labeling was also seen in the mid-portion of the inner dental epithelium in areas where enamel knots were not observed.

◀ **Fig. 1A–H** Transverse sections of developing human teeth at weeks 9, 10 and 11. In situ hybridization with ^{35}S-labeled human NGF and BDNF mRNA probes. Sections were photographed under dark- or bright-field illumination. Bright-field photographs **(A, C, E, G)** visualize the structures studied. **A, B** NGF mRNA labeling is found in the dental papilla at 9 weeks. **C, D** BDNF mRNA labeling is found lateral to the tooth anlagen (i.e., lateral to the outer dental epithelium) at 9 weeks (*arrow*). Labeling starts at the vestibular lamina (*filled arrow*) and extends laterally to the lateral side of the dental follicle. Labeling is also seen in the area of the enamel knot (*open arrow*) towards the inner dental epithelium. **E, F** BDNF mRNA labeling at 10 weeks. Strong labeling is observed in the area of the cervical loop (*filled arrow*). Labeling is also observed in the mesenchyme located lateral to the developing teeth (*arrowhead*). **G, H** BDNF mRNA labeling at 11 weeks in the inner dental epithelium (*filled arrow*). *Scale bar in* **B** represents 125 μm and applies to all parts

At 11 weeks of gestation (Fig. 1G, H), BDNF labeling showed a similar expression pattern to that of earlier stages. The labeling in the subepithelial mesenchyme was, however, below the detection level in the upper and lower jaws. The mesenchyme lateral to the developing teeth continued to express BDNF mRNA. Labeling was observed throughout the dental follicle, extending from the lateral part of the outer enamel epithelium to the medial part. Labeling was seen in the medial parts of the inner dental epithelium, and appeared to be stronger at 11 weeks compared to prior stages.

Comparative aspects

Oral subepithelial labeling at 10 weeks of gestation had decreased compared to that of 9 weeks. The general mesenchymal labeling lateral to the developing teeth and dental lamina observed at 9 weeks had also decreased at 10 weeks and was seen in small restricted areas lateral to the outer dental epithelium (i.e., had both decreased labeling and extension of the labeled areas). The labeling was below the detection level in the lateral side of the outer dental epithelium and presumably shifted towards the cervical loop in the lateral part of the outer dental epithelium and the medial part of the inner dental epithelium at 10 weeks. A stronger labeling was observed on the medial side of the inner dental epithelium, in the dental follicle and in the enamel knot at 10 weeks compared to 9 weeks.

NT-3 mRNA

At 6.5 weeks of gestation, broad NT-3 mRNA labeling was observed in the anterior parts of the developing maxillary and mandibular processes. Moderate to strong NT-3 labeling was detected at 7 weeks in the dental papilla and in the oral epithelium. At 7.5 weeks, NT-3 labeling was still present in the oral epithelium and in the subepithelial mesenchyme. At 9 weeks of gestation, NT-3 mRNA labeling was observed in the basal layer of the oral epithelium and in the adjacent subepithelial mesenchyme. In addition, labeling was seen in the mesenchyme lateral to the developing teeth and dental follicle, extending to the dental papilla, and in the basal layer of the dental lamina of developing teeth (Fig. 2A, B). Dental papillae NT-3 mRNA labeling was stronger than that of BDNF mRNA labeling, but was weaker than that of BDNF mRNA labeling in other areas. NT-3 mRNA labeling was not seen in the enamel knot at this stage. At 10 weeks, a strong labeling was clearly observed in the dental papillae. In the anterior teeth, dental papilla labeling appeared weaker than that of posterior teeth. Labeling was observed in the epithelial transitional area between the inner and outer dental epithelium, in the area of the cervical loop, mainly in the molar teeth. NT-3 mRNA labeling was still found in the basal layer of the oral epithelium and in the subepithelial mesen-

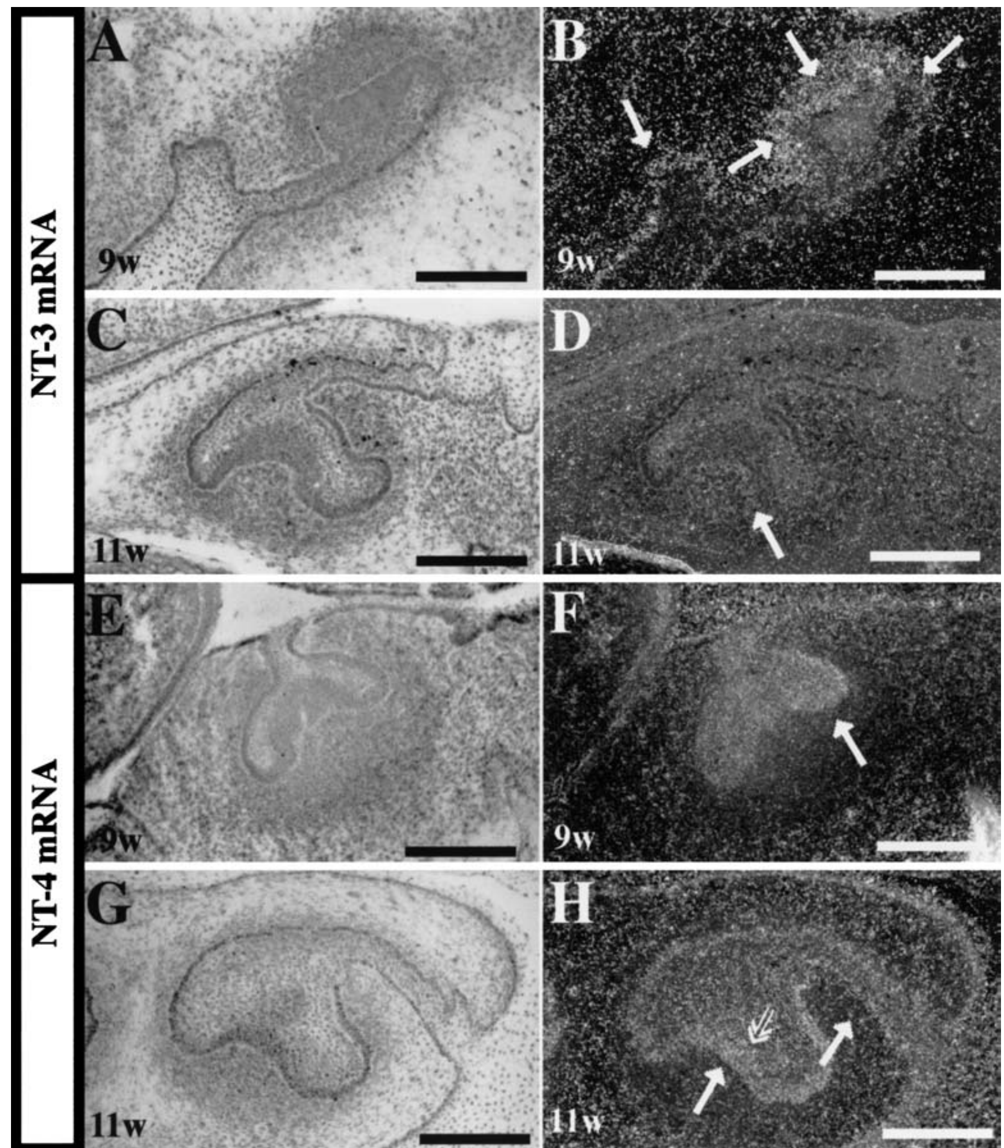


Fig. 2A–H Transverse sections of developing human teeth at weeks 9 and 11. In situ hybridization with ^{35}S-labeled human NT-3 and NT-4 mRNA probes. Sections were photographed under dark- or bright-field illumination. **A, B** At 9 weeks, NT-3 mRNA labeling (*arrows*) is observed in the mesenchyme lateral to the tooth anlagen, in the dental follicle, extending to the dental papillae, and in the basal layer of the dental lamina of the developing teeth. **C, D** At 11 weeks, NT-3 mRNA labeling (*arrow*) is seen in the dental papillae (however, it is weaker than that at 9 weeks). **E, F** At 9 weeks, NT-4 mRNA labeling is observed in the oral epithelium, dental lamina, inner and outer dental epithelium, and the stellate reticulum (*filled arrow*). **G, H** At 11 weeks, labeling is also observed in the enamel knot (*open arrow*) in addition to the labeled structures observed at prior stages (*filled arrows*). *Scale bars* 250 μm

chyme anteriorly (where incisor and canine teeth are located), but the subepithelial labeling was below detection level in the posterior areas (in the area of the molar teeth).

At 11 weeks of gestation (Fig. 2C, D), NT-3 mRNA labeling was weaker than at 10 weeks. The expression was below detection level in the basal layer of the oral epithelium and was not seen in the subepithelial mesenchyme. The labeling in the mesenchyme lateral to the developing teeth was weaker than at prior stages. The labeling in the lateral part of the dental follicle was below detection level and the enamel knot did not show any NT-3 labeling at this stage. Dental papillae labeling in both anterior and posterior teeth was weaker than at 10 weeks. NT-3 labeling in the medial part of the cervical loop was below detection level, but weak labeling was observed in the lateral part of the cervical loop.

Comparative aspects

Dental papillae labeling at 10 weeks of gestation was more prominent than that at 9 weeks. NT-3 mRNA labeling was also clearly visible in the area of the enamel knot.

NT-4 mRNA

At 6.5 weeks of gestation, strong NT-4 mRNA labeling was detected in the oral epithelium. Such strong labeling was found at 7 weeks in the oral epithelium and in the dental lamina and developing teeth. At 8.5 weeks, labeling was observed in the lateral parts of the outer and inner dental epithelia as well as in the tongue epithelium.

At 9 and 10 weeks of gestation (Fig. 2E, F), strong labeling was observed in the outer and inner dental epithelia. Strongest labeling was seen in the lateral side of the

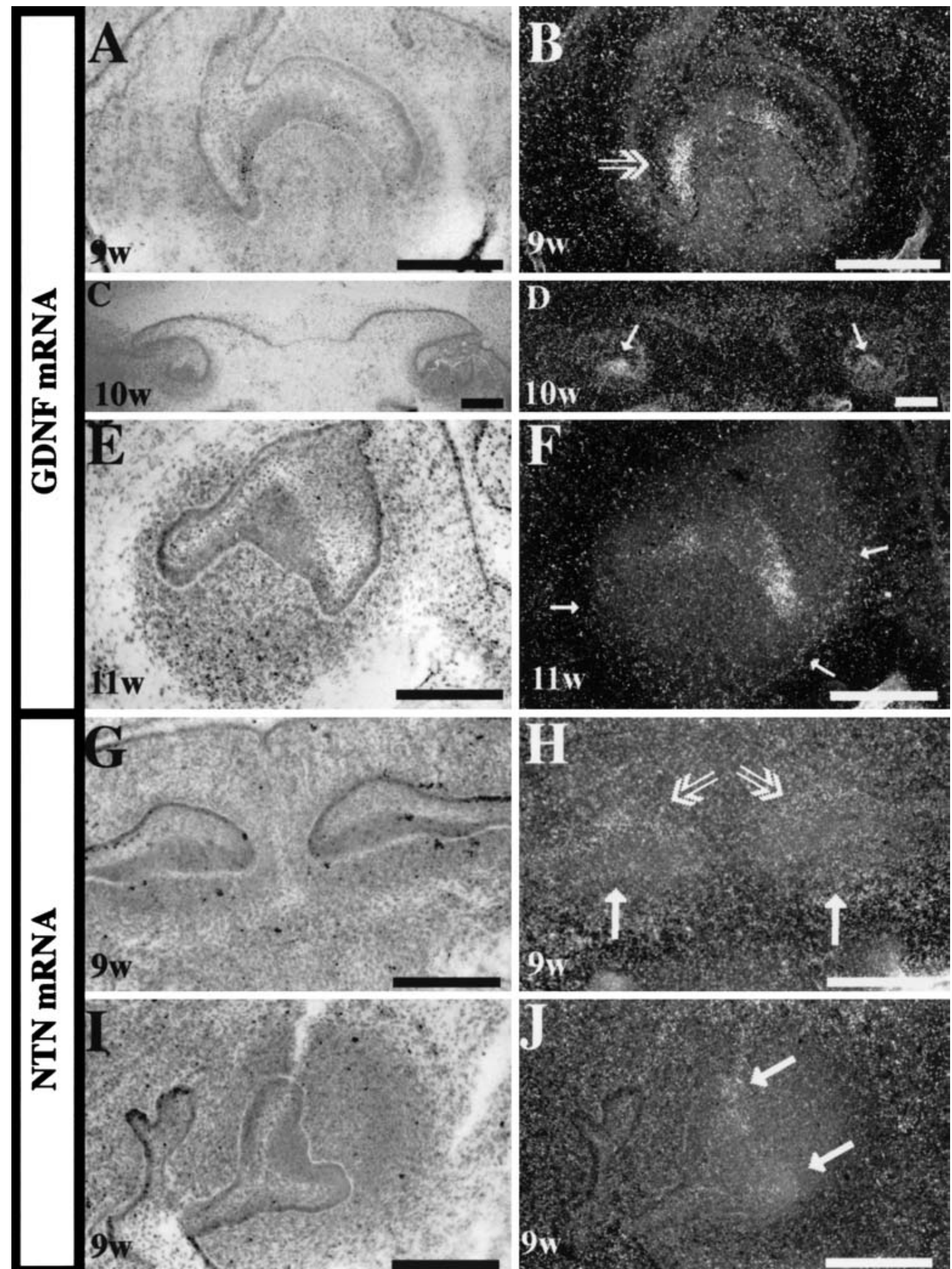


Fig. 3A–J Transverse sections of developing human teeth at weeks 9, 10 and 11. In situ hybridization with ^{35}S-labeled human GDNF and NTN mRNA probes. Sections were photographed under dark- or bright-field illumination. **A, B** At 9 weeks, GDNF mRNA is observed in the medial side (*open arrow*), and to a lesser extent in the lateral side of the inner dental epithelium of the developing teeth. **C, D** Two anterior developing human teeth at 10 weeks. GDNF mRNA labeling is mainly observed in the area of the enamel knot (*arrows*). **E, F** At 11 weeks, GDNF mRNA labeling is seen in medial (stronger labeling), lateral parts of the inner dental epithelium, and in the dental follicle (*arrows*). **G, J** At 9 weeks, NTN mRNA labeling is observed in the inner (*solid arrows*) and outer (*open arrows*) dental epithelia and in the cervical loop area (*arrows in* **J**). *Scale bars* 250 μm

cervical loop. Oral epithelium showed NT-4 mRNA labeling, but not to the same extent as dental epithelium. NT-4 mRNA expression was also seen in the dental lamina and stellate reticulum. At 10 weeks, basal layers of the epithelium generally contained stronger labeling than other epithelial layers. At 11 weeks (Fig. 2G, H), labeling was also observed in the enamel knot.

GDNF mRNA

At 6.5 weeks of gestation, labeling was observed around several facial cartilages. A subepithelial labeling was observed in the anterolateral parts of the tongue. It was strongest in the ventral part of the mesenchyme. In the posterior region, where the palatal shelves had fused, broad subepithelial labeling was seen. At 7 weeks, the subepithelial labeling of the tongue was weaker than that at 6.5 weeks. Strong labeling was seen around Meckel's cartilage. At 8.5 weeks of gestation, labeling was seen on the medial side of the dental follicle and the parts adjacent to the dental papillae. Labeling was also observed in the dental papillae. Strong labeling was seen in the mesenchyme of the ventral lingual epithelium.

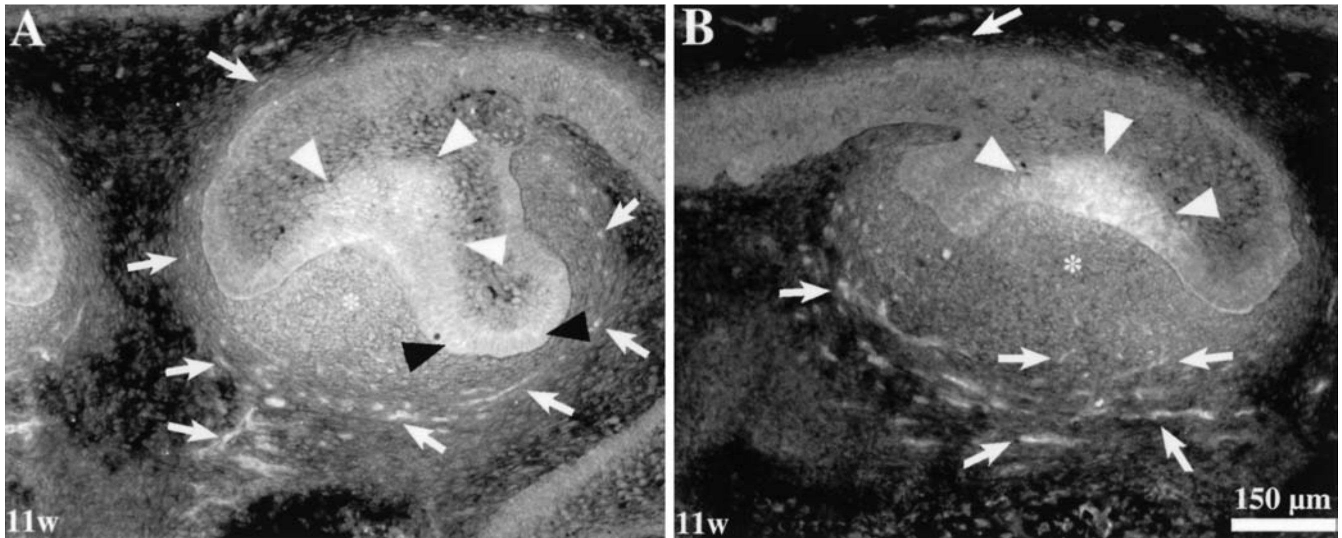


Fig. 4A, B Transverse sections of developing human teeth at 11 weeks. Photomicrographs visualize protein gene product (PGP) 9.5-immunoreactive nerve fibers in the area of the developing teeth. PGP-positive nerve fibers are found in the dental follicle of the developing teeth (*arrows*) and around the teeth. PGP-positive areas are also observed in the inner dental epithelium/enamel knot (*white arrowheads in* **A** *and* **B**) and in the cervical loop area (*black arrowheads in* **A**). *Scale bar* 150 µm

At 9 weeks, labeling was observed in the dental follicles. Strong labeling was shown in the medial and lateral sides of the inner dental epithelium (Fig. 3A, B). The labeling was also observed subepithelially in the anterior parts of the oral cavity. At 10 weeks of gestation, labeling was also observed in the enamel knot (Fig. 3C, D). The medial side labeling of the inner dental epithelium was stronger than that of the labeling in the lateral side. The labeling in the dental follicle extended medially.

At 11 weeks (Fig. 3E, F), labeling was seen in the entire dental follicle. Strongest labeling was seen on the medial side, and the weakest on the lateral side. The medial part of the inner dental epithelium contained strong labeling, while the labeling in the lateral part was weaker. Labeling was also detected in the enamel knot. Subepithelial mesenchymal cells in the anterior part of the oral cavity contained GDNF labeling, similar to that seen at prior stages.

Comparative aspects

Labeling was observed in the lateral part of the inner dental epithelium and enamel knot at 10 weeks of gestation. These areas were not labeled at 9 weeks. Labeling in the dental follicle was also stronger at 10 weeks compared to 9 weeks.

NTN mRNA

At 6.5 weeks of gestation, labeling was seen in the oral and lingual epithelia. At 8.5 weeks, weak labeling was observed in the lingual epithelium and lateral aspects of the dental lamina. At 9 and 10 weeks (Fig. 3G, J), NTN mRNA labeling was seen in the subepithelial mesenchymal cells in the anterior part of the oral cavity. Labeling was seen in the inner dental epithelium and in the area of the cervical loop. However, at 11 weeks of gestation, NTN mRNA labeling in oral structures was below expression level.

Immunohistochemistry

We used antibodies to PGP 9.5 to localize nerve fibers at 11 weeks to gain an understanding of the extent of innervation of developing teeth at the oldest developmental stage studied here. PGP-positive nerve fibers were in close proximity to developing teeth (Fig. 4A, B). Small bundles were found in the future apical location of teeth, outside the dental follicle, and would branch off to smaller nerve fibers that extend into the dental follicle. PGP-positive nerve fibers were located in the dental follicle around the teeth. PGP-positive areas were also observed in the inner dental epithelium and in the area of the cervical loop, the significance of which is not known to us.

Discussion

In the present study, we show distinct and developmentally shifting patterns of expression for several neurotrophic factors in the developing human teeth. NGF mRNA labeling level was weak and confined to the dental papillae. Dental BDNF mRNA labeling was strong and found in several different areas, such as in the mesenchyme lateral to the developing teeth, the inner dental epithelium, and the enamel knot. NT-3 mRNA expression was found predominantly in the dental papilla. GDNF mRNA was found in the inner dental epithelium and in the enamel knot. In addition, NT-4 mRNA labeling was found in

epithelially derived tissue compartments, i.e., in the dental epithelia as well as in the oral epithelium.

In rodents, neurotrophin mRNA labeling is generally weak in prenatal stages (see Luukko et al. 1997a; Nosrat et al. 1997b). However, strong pulpal mRNA labeling for NGF, BDNF and GDNF is found postnatally prior to the initiation of dental pulp innervation (Mohamed and Atkinson 1983) and persists for several days after that initiation (Luukko et al. 1997a; Nosrat et al. 1997b). In rats, BDNF and GDNF mRNA labeling was rather weak at prenatal stages and became distinct only at postnatal ages (Nosrat et al. 1997b). Developing human teeth in the present study represent early stages of tooth development, from bud to cap stage, and correspond to early prenatal stages of tooth development in rodents. However, the findings here clearly show that BDNF and GDNF mRNA are expressed at higher levels in developing human teeth compared to rodents at these early stages and that NGF expression is weaker in the developing human teeth than in rodent teeth. This suggests additional and possibly somewhat overlapping roles for neurotrophins and GDNF in the developing human teeth. It also indicates a clear difference between species regarding the expression patterns of neurotrophic factor mRNA in developing teeth. Similar differences between rodents and humans have also been observed in the gustatory system. While BDNF and NT-3, two neurotrophins that are involved in initiation and maintenance of gustatory and somatosensory innervation of the tongue in rodents, are distinctively located to either taste buds or somatosensory-related areas in the rodent tongue (Nosrat et al. 1996a, 1997a), they have broader and somewhat overlapping expression patterns in developing and adult human tongue (Nosrat et al. 2000).

In humans, the dental follicle becomes innervated in the early cap stage, while there is a delay in dental papillae innervation until dentin and enamel matrices are formed (Christensen et al. 1993). In the present study, we demonstrated that PGP-positive nerve fibers were present only in the dental follicles of the developing teeth at 11 weeks of gestation, the oldest stage studied. In addition, we also showed that BDNF and GDNF mRNAs were present in the dental follicle during the time when dental follicles became innervated, and therefore might be involved in tooth innervation also in humans.

There are no data regarding expression of trophic factor receptors in human teeth. In rats, some neurotrophin receptors and receptor components for the GDNF family of ligands are found in developing teeth. The receptor components for both neurotrophins and GDNF have also been found in the trigeminal ganglion. GDNF is also retrogradely transported to a population of trigeminal neurons, mainly large-size neurons coexpressing both ret and GFRα-1, suggesting a neurotrophic role for GDNF in the trigeminal system (Nosrat et al. 1997c). We have also shown that dental pulp cells provide neurotrophic support for trigeminal neurons in vitro, indicating a specific role for dental-pulp-derived neurotrophic factors in dental pulp-trigeminal neuron interactions (Nosrat et al. 2001).

Conventional gene-knockouts allow studies of the consequences of development in the absence of a given protein. It has been shown that the dental pulp in NGF-heterozygous mice has only 10–30% of the normal innervation while the dental pulp totally lacks innervation in NGF-knockout mice (Byers et al. 1997). Interestingly, the periodontal and gingival innervation are also reduced in these animals (Byers et al. 1997). It was recently shown that the dental pulp is also devoid of any nerve fibers in trkA knockout mice (Matsuo et al. 2001). Despite the obvious importance of NGF-signaling in tooth innervation, NGF mRNA labeling was rather weak in the developing human deciduous teeth. This suggests the possibility that BDNF and NT-3, which are highly expressed in developing human teeth, are substituting for the early roles of NGF. It is also possible that there is a difference in the pattern of expression of NGF mRNA in deciduous and permanent teeth. Nevertheless, weak NGF labeling was observed in the developing deciduous teeth.

While gross tooth morphology appears normal in GDNF knockout mice (Granholm et al. 1997; de Vicente et al. 2002), detailed analysis has revealed that both odontoblasts and ameloblasts fail to fully develop and differentiate (de Vicente et al. 2002). Interestingly, enamel matrix and predentin layers were also absent in GDNF knockout mice compared to controls (de Vicente et al. 2002). The data are intriguing, but further studies are required. Specifically, it needs to be clarified whether these deficits are primary or due to agenesis and non-functionality of the kidneys and the enteric nervous system. Agenesis and non-functionality of the kidneys lead to metabolic imbalance and increased levels of toxic metabolites such as polyurea. It is therefore imperative to verify the findings in prenatal animals to minimize metabolic influences on odontogenic cell survival and morphology. Lack of secreted components (i.e., enamel matrix and predentin) in GDNF knockout mice is, however, a good indication that the deficits are local rather than general. Despite the lack of information on tooth development and morphology in neurotrophin knockout animals, it seems that gross tooth morphology is not affected in neurotrophin knockouts (our unpublished data). However, detailed studies are required to verify this. In addition, there is functional redundancy among the neurotrophic factor family members. Most importantly, all of these factors are expressed in the developing teeth. One cannot exclude the morphogenic influences of neurotrophic factors on tooth development before knockout mice with combinatory deletions of neurotrophic factor genes are studied in detail. The concept of excluding the importance of a factor based on a single-gene null mutation is not reliable, as clearly indicated by a rather normal gross morphology of the CNS in neurotrophic factor knockout mice and by retinoic acid and orphan receptor knockout mice (Krezel et al. 1996; Subbarayan et al. 1997; Li et al. 2000). The effects of a single neurotrophic factor gene deletion could be masked by the presence of the other family members in the developing teeth.

Our findings might have clinical implications by opening new possibilities for treatment of neurodegenerative diseases and/or nerve injury. Cells or tissues from fetal and/or possibly adult teeth might offer trophic support for remaining or transplanted cells, a strategy that has been widely suggested, studied and used in both human patients and animal models (see Hefti et al. 1989; Olson 1993). Indeed, it has been shown that co-grafting kidney tissue together with ventral mesencephalic grafts not only enhances fiber outgrowth into the 6-hydroxydopamine-lesioned striatum, but also improves behavioral recovery (Granholm et al. 1998). Similarly, Sertoli cells, another rich source of trophic factors, have been grafted to the brain to counteract symptoms of neurotoxic lesions of the nigrostriatal dopamine system (Borlongan et al. 1997; Sanberg et al. 1997). Preservation of teeth in the older human population would increase availability of dental tissues for possible uses as allografts.

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