

Neurotrophins in odontogenesis

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ABSTRACT Neurotrophins (NTFs) are a family of structurally related proteins with specific effects on the developing nervous system and a wide range of non-neuronal differentiating cells. To date, four NTFs have been characterized: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). To perform their biological effects, the NTFs must bind to appropriate receptors on the surface of responsive cells. High- and low-affinity receptors for NTFs have been identified. The high-affinity receptors are members of the *trk* protein tyrosine kinase receptor family. The low-affinity neurotrophin receptor *gp75^{NTFR}* is a common receptor for all NTFs. Here we summarize some of our previous findings on the expression patterns of NGF, *gp75^{NTFR}*, *TrkB*, and *TrkC* in the developing molar tooth of the rat. Both NGF and *gp75^{NTFR}* are localized in dental epithelium and mesenchyme but often their expression patterns differ. Concomitant expression of NGF and *gp75^{NTFR}* in mesenchyme is correlated with odontoblast differentiation. The *trkB* and *trkC* receptors show distinct cell-specific expression patterns in developing tooth, suggesting that other NTFs, apart from NGF, may be involved in odontogenesis. These data demonstrate that NTFs participate in the cascade of molecular events that direct tooth development, and support the notion that NTFs may have multiple and distinct roles in dental tissues.

KEY WORDS: *neurotrophins, trk, tooth, morphogenesis, differentiation*

Introduction

Tooth development results from a complex set of secondary interactions between the oral ectoderm and the neural crest-derived ectomesenchyme (Thesleff and Hurmerinta, 1981). Attempts to determine the tissue responsible for inducing tooth formation have given conflicting results, suggesting that the odontogenic potential resides either in oral epithelium or in mesenchyme (reviewed by Ruch, 1987, 1995). More recently, Mina and Kollar (1987) and Lumsden (1988) pointed out the importance of oral epithelium in tooth induction. The role of innervation in tooth development has also been the subject of intense investigation at the level of experimental embryology. Previous studies on mouse embryos indicated that both tooth initiation and morphogenesis are independent of innervation (Lumsden and Buchanan, 1986). However, recently Tuisku and Hildebrand (1994) demonstrated that the participation of nerve fibers is a prerequisite for tooth formation in a polyphyodont teleost (cichlid *Tilapia mariae*). Nerve fibers reach the subepithelial mesenchyme at future sites of tooth development before the formation of tooth primordia (Kollar and Lumsden, 1979), suggesting that the presumptive dental epithelium and/or mesenchyme may express diffusible molecules attracting the growing nerve axons.

Diffusible factors regulating both proliferation and differentiation of neuronal precursors may additionally act as chemoattractant molecules, directing nerve fibres to their specific targets (Gunderson

and Barrett, 1979; Davies *et al.*, 1987; Cattaneo and McKay, 1990; Rawdon, 1991). Nerve growth factor (NGF) is the first characterized of the diffusible factors with neurotrophic activities. NGF supports the survival and outgrowth of selected groups of neurons *in vivo* and *in vitro* (reviewed by Levi-Montalcini, 1987; Thoenen *et al.*, 1987). Brain-derived neurotrophic factor (BDNF) is another neurotrophic factor initially isolated from pig brain (Barde *et al.*, 1982) with about 50% amino acid similarity to NGF (Leibrock *et al.*, 1989) and which supports the survival of other neuronal populations (reviewed by Snider, 1994). Based on the sequence similarity, additional members of a family of structurally related genes (called NGF-related neurotrophins) have been isolated coding for neurotrophin-3 (NT-3) (Maisonpierre *et al.*, 1990) and neurotrophin-4 (NT-4) (Hallböök *et al.*, 1991), also named neurotrophin-5 (NT-5) (Berkemeier *et al.*, 1991).

The biological effects of neurotrophins (NTFs) are mediated by specific receptors on the surface of the responsive cells. Two binding affinities of NTFs to their appropriate receptors, one high, the other low, have been described. The neurotrophin receptor (NTFR) *gp75^{NTFR}* is a transmembrane glycoprotein which has no identifiable catalytic activity in its cytoplasmic region and binds all NTFs at low-

Abbreviations used in this paper: NT-3, neurotrophin-3; NT-4, neurotrophin-4; TGF β 1, transforming growth factor β 1; NTFs, nerve growth factors; BDNF, brain-derived neurotrophic factor.

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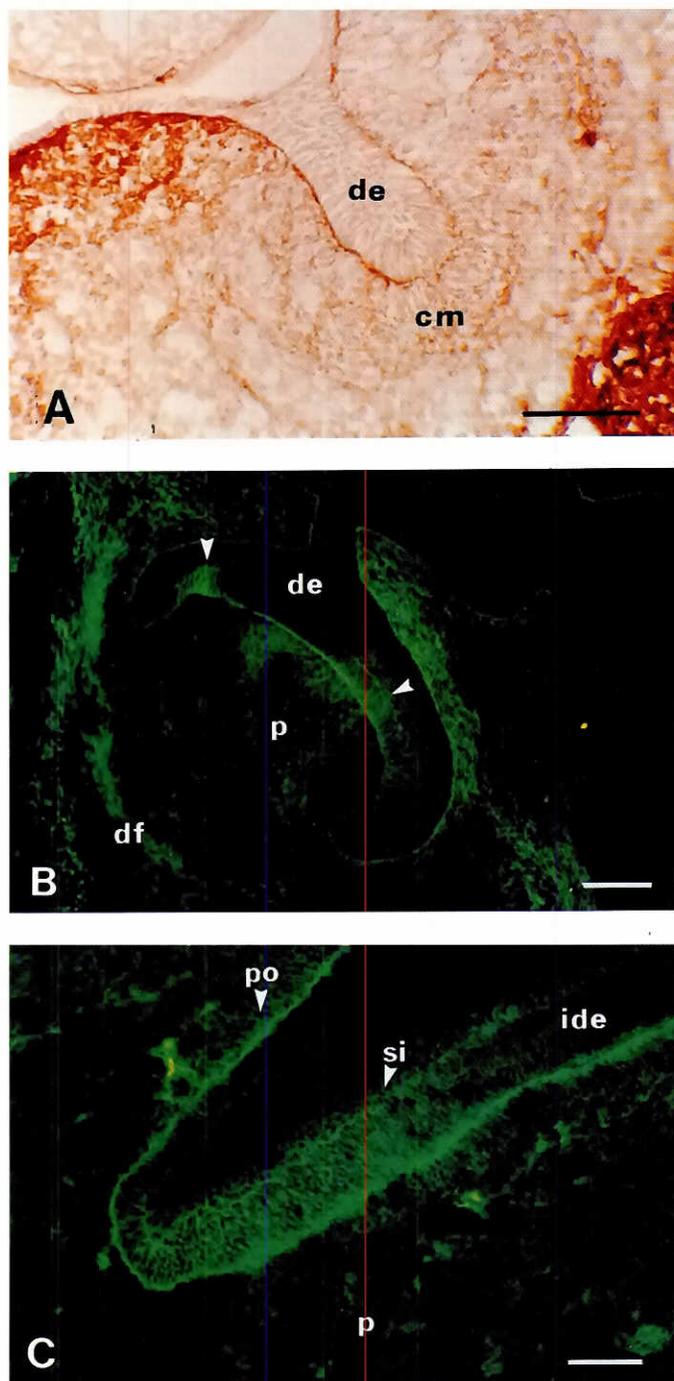


Fig. 1. Immunohistochemical localization of the low-affinity neurotrophin receptor gp75^{NTFR} in the developing first molar of the rat. (A) Bud staged tooth (E15). Staining is absent from dental epithelium (de), whereas the condensed mesenchyme (cm) exhibits faint immunoreactivity. Mesenchymal cells adjacent to the basement membrane are also stained. (B) In the cap staged tooth (E17), gp75^{NTFR} staining is detected in cells of the inner dental epithelium and stratum intermedium (arrowheads), in dental papilla (p) and dental follicle (df). de, dental epithelium. (C) Dental groove area (fissure) of a molar at the bell stage (E21). gp75^{NTFR} staining is found on cell surfaces of inner dental epithelial cells (ide), some cells of the stratum intermedium (si), and in polarizing odontoblasts (o). p, dental papilla. Scale bars, 50 μ m.

affinity (Radeke *et al.*, 1987; Chao, 1992). The products of the tyrosine kinase *trk* family of proto-oncogenes also bind NTFs, and are components of the high-affinity receptor. The *trk* gene family consists of three characterized genes, *trkA*, *trkB*, *trkC*, and additional uncharacterized members (reviewed by Barbacid *et al.*, 1991; Chao, 1992; Parada *et al.*, 1992; Jelsma and Aguayo, 1994; Johnson and Oppenheim, 1994). The *trkA* gene encodes a glycoprotein of about 140 kDa (TrkA or gp140^{trk}) with a tyrosine kinase activity, which functions as a NGF receptor (Klein *et al.*, 1991). BDNF and NT-4 are the preferred ligands for TrkB (or gp145^{trkB}) (Squinto *et al.*, 1991; Klein *et al.*, 1992) and NT-3 for TrkC (or gp145^{trkC}) (Lamballe *et al.*, 1991). Alternative transcripts for Trk receptors have been identified including receptors that lack functional kinase domains (truncated receptors of TrkB and TrkC). The role of gp75^{NTFR} in the formation of high-affinity receptors and in the signal transduction is controversial (reviewed by Chao, 1992; Heumann, 1994). Functional high-affinity neurotrophin binding appears to require either co-expression and binding to both gp75^{NTFR} and Trk receptors (Hempstead *et al.*, 1991; Verdi *et al.*, 1994), or binding to dimers of Trk receptors (Barbacid *et al.*, 1991; Klein *et al.*, 1991).

The view on the role for NTFs in embryonic development has changed from being strictly involved in target to neuron trophic interactions to include roles in organ morphogenesis and non-neuronal cell differentiation (Aloe and Levi-Montalcini, 1977; Matsuda *et al.*, 1988; Otten *et al.*, 1989). The novel roles for NTFs are also suggested by the presence of gp75^{NTFR} and Trk receptors in developing embryonic tissues and organs (Yan and Johnson, 1988; Ernfors *et al.*, 1992). Information concerning the expression of NGF and gp75^{NTFR} in the developing and injured rodent teeth has emerged the last few years (Yan and Johnson, 1988; Byers *et al.*, 1990, 1992a,b; Mitsiadis *et al.*, 1992, 1993a,b,c; Mitsiadis, 1993). Some of these findings are reported in the present review, whereas new information is given on the *trkB* and *trkC* mRNA expression in the developing rodent tooth (Luukko *et al.*, submitted). Furthermore, we report some experimental data on cultured tooth germs *in vitro* in the presence or absence of NGF. These findings offer evidence for the participation of NTFs in tooth morphogenesis and differentiation, and contribute to the understanding of NTFs action in development in general.

Results

Immunolocalization of gp75^{NTFR} and NGF in the developing molar of the rat

During the initiation of tooth development (E13), gp75^{NTFR} immunoreactivity was absent in both thickened oral epithelium and subjacent mesenchyme, whereas weak NGF-like immunoreactivity was observed in the epithelium. In the E15 rat embryo, gp75^{NTFR} staining was absent from dental epithelium of the bud staged molar, but a faint reactivity was found in the condensed mesenchyme (Fig. 1A). Weak NGF-like immunoreactivity was observed in both dental epithelium and mesenchyme. In the cap staged tooth (E17), gp75^{NTFR} staining was observed in parts of the inner dental epithelium and stratum intermedium, in dental papilla and follicular mesenchyme (Fig. 1B). NGF-like immunoreactivity was evident in dental epithelium, whereas the staining was very faint in mesenchyme (Fig. 2A). At the advanced bell stage (E21-PN1), gp75^{NTFR} staining in dental epithelium was evident in inner dental epithelium and some cells of the stratum intermedium, whereas the reactivity was absent from preameloblasts before their terminal differentiation in ameloblasts (Fig. 1C). Polarizing odontoblasts exhibited gp75^{NTFR} reactivity,

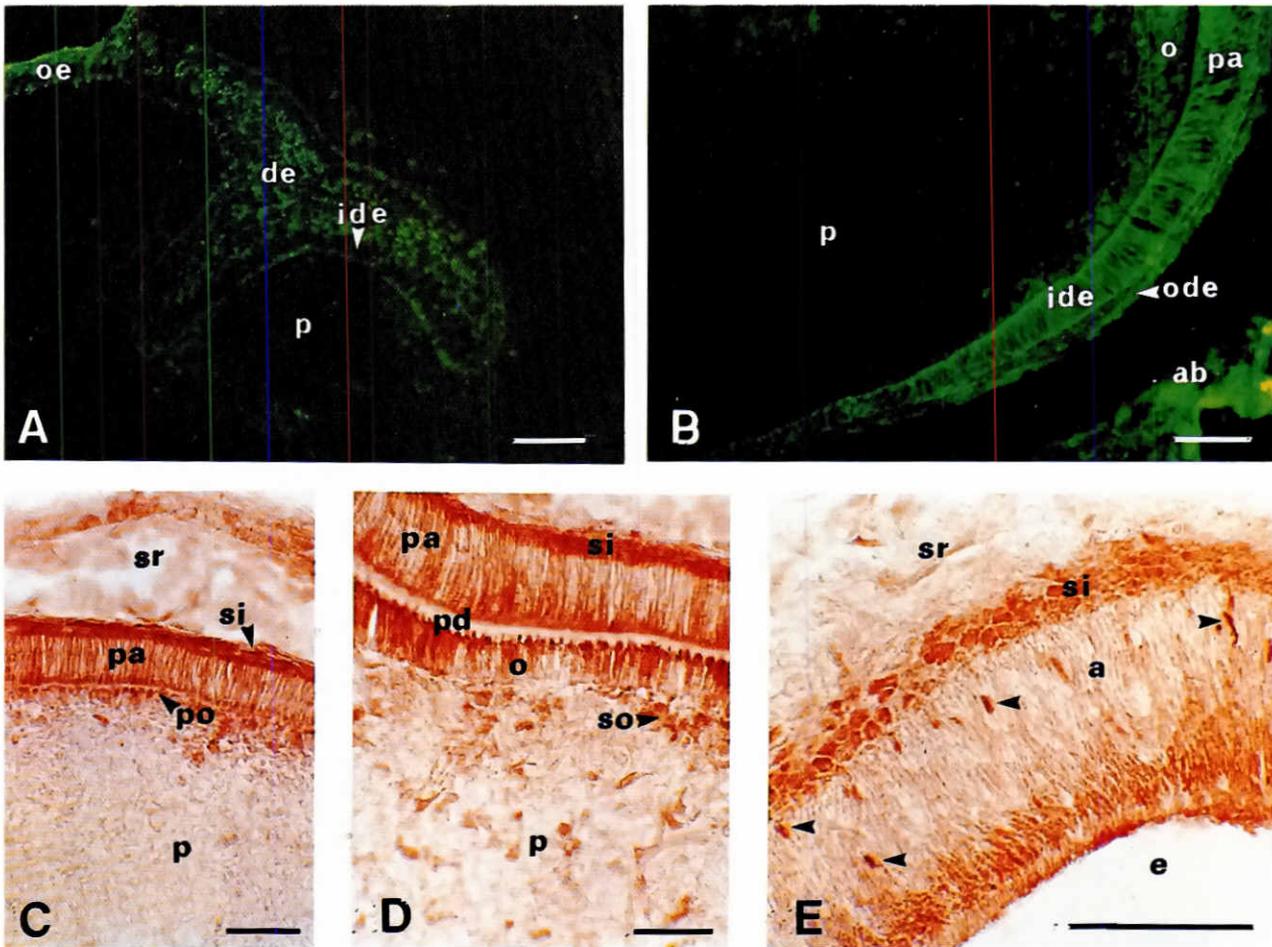


Fig. 2. Detection of NGF-like immunoreactivity in the developing first molar of the rat. (A) At cap stage (E17), NGF staining is found in the central part of the dental epithelium (de). The staining was moderate in cells of the outer and inner dental epithelium (ide, arrowhead). In dental papilla (p) the staining was very faint. oe, oral epithelium. (B) High magnification of the cervical loop area of a E21 tooth germ. NGF staining is found in all epithelial layers of the tooth, as well in polarizing odontoblasts. (C, D, and E) Distribution of NGF during odontoblast and ameloblast differentiation (from E21 to postnatal day 5). Intense staining is observed in stratum intermedium (si), preameloblasts (pa), ameloblasts (a), polarizing odontoblasts (po), differentiated odontoblasts (o), and cells of the sub-odontoblastic layer (so). Note the strong staining in nerve fiber-like structures localized between ameloblasts (arrowheads in E). sr, stellate reticulum; ide, inner dental epithelium; ode, outer dental epithelium; p, dental papilla; ab, alveolar bone; pd, predentin; e, enamel. Scale bars, 50 μ m.

whereas functional odontoblasts, located at the tip of the cusps, were gp75^{NTR}-negative. NGF-like immunoreactivity was detected in all layers of dental epithelium (Fig. 2B,C and D), but the strongest staining was observed in stratum intermedium. In dental papilla, the staining first appeared in polarizing odontoblasts (Fig. 2B and C) and persisted in functional odontoblasts (Fig. 2D). At PN4, gp75^{NTR} staining was detected in cells of the sub-odontoblastic layer and proliferating cells of the inner dental epithelium, whereas NGF-like immunoreactivity was observed in ameloblasts, stratum intermedium, odontoblasts and cells of the sub-odontoblastic layer (Fig. 2E). Strong NGF staining was found in neuronal-like structures located in the layer of ameloblasts (Fig. 2E, arrowheads).

The distribution patterns of gp75^{NTR} and NGF in the developing first molar tooth are schematically summarized in Figure 5.

Expression of *trkB* and *trkC* transcripts in the developing molar of the rat

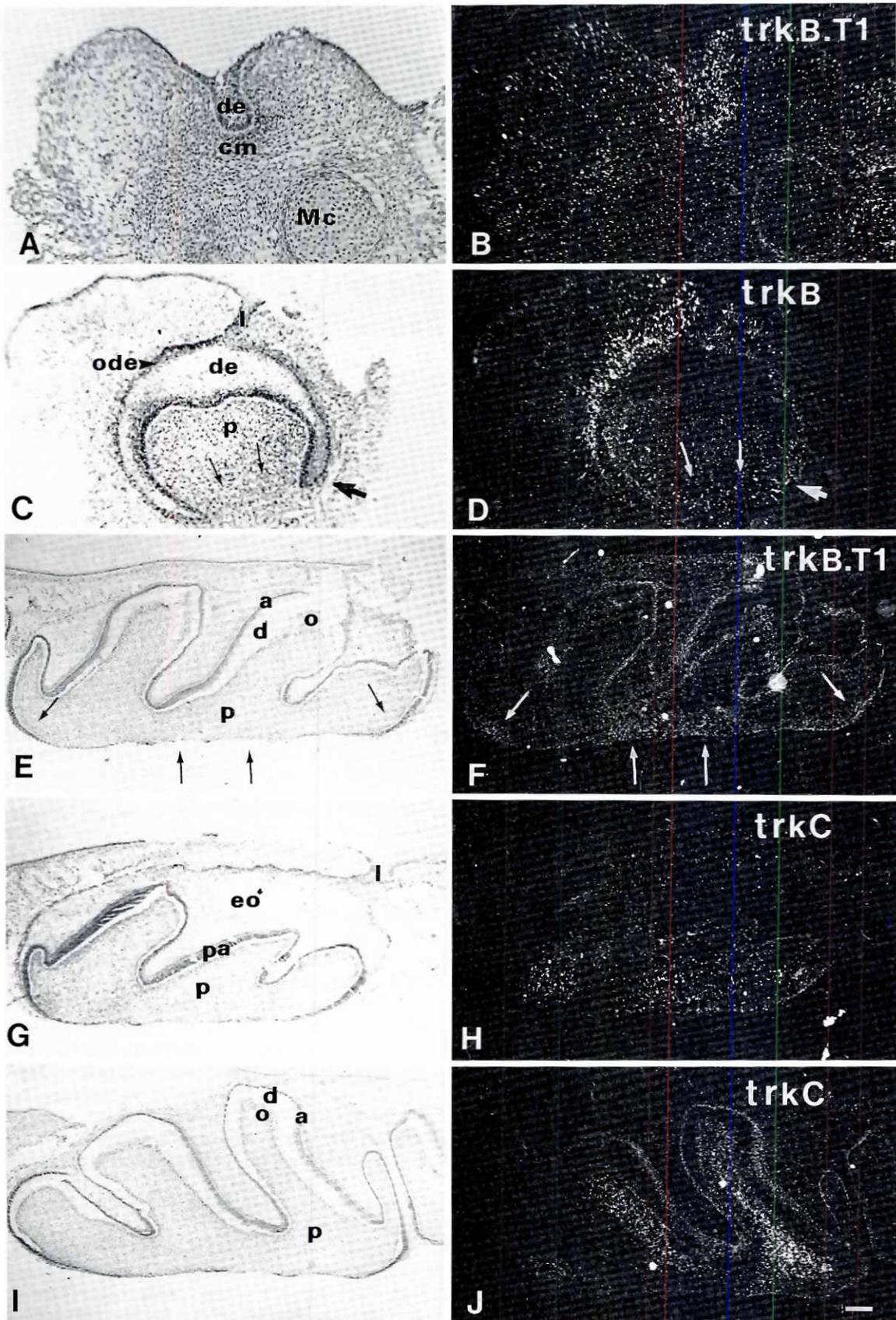
The expression of *trkB* and *trkC* transcripts were studied by *in situ* hybridization. At E14-E15, the truncated form of the *trkB.T1* of

the receptor was expressed in both dental epithelium and mesenchyme (Fig. 3A and B). At the early bell stage (E19), *trkB* transcripts were detected in outer dental epithelium, in epithelium and mesenchyme of the cervical loop area, and in mesenchyme lining the dental lamina and the cervical part of the outer dental epithelium (Fig. 3C and D). At PN4, *trkB.T1* transcripts were expressed in the lower part of the dental papilla and in mesenchyme adjacent to epithelium of the cervical loop area (Fig. 3E and F).

trkC mRNA were not detected in dental tissues before PN1. At PN1, transcripts were found in dental papilla mesenchyme, but not in regions lining the inner dental epithelium (Fig. 3G and H). At PN4, the hybridization signal increased in dental papilla (Fig. 3I and J).

Effects and distribution of NGF in mouse tooth germs cultured as explants *in vitro*

E16-E17 mouse molars are at the bell stage and cusps have begun to develop. E16 tooth germ explants cultured in RPMI (a serum-free medium) for 6 days demonstrated normal cusp formation containing functional odontoblasts and ameloblasts. The pres-



ence of NGF (50-100 ng/ml) in the medium did not affect morphogenesis and cytodifferentiation (Fig. 4A), but the tooth germs were smaller than normal (data not shown).

Immunohistochemistry was performed in sections from these explants. In explants cultured in RPMI, strong NGF-like immunoreactivity was detected in the stellate reticulum (data not shown), whereas in explants supplemented with NGF the staining was localized in functional odontoblasts (Fig. 4B). Sections from E16 tooth germs cultured in a medium supplemented with 20% fetal calf serum demonstrated the same distribution patterns of NGF-like immunoreactivity as in the *in vivo* developing tooth (data not shown).

Discussion

In this paper we describe the expression patterns of nerve growth factor (NGF), low-affinity neurotrophin receptor gp75^{NTFR}, *trkB*, and *trkC* in the developing first molar of the rat. NGF, gp75^{NTFR}, and *trkB* are expressed during histo-morphogenesis and cytodifferentiation, whereas *trkC* is expressed at more advanced developmental stages. The sequential chain of modifications in NGF and gp75^{NTFR} expression coincides with epithelial-mesenchymal interactions that occur during the different stages of tooth development (Fig. 5). The implication of NTFs in tissue-tissue interactions has been also suggested for the developing skin and whisker follicles (Davies *et al.*, 1987; Yaar *et al.*, 1991). The temporospatial distribution of NGF and gp75^{NTFR} in the developing rat tooth is very similar to that observed in the deciduous human teeth (Mitsiadis, 1993; Mitsiadis *et al.*, 1993c; T. Mitsiadis and D. Maquin, unpublished results), indicating that the molecular mechanisms controlling odontogenesis have been conserved during evolution.

Neuronal functions for NTFs

During embryonic development, the expression of NGF, gp75^{NTFR}, and *trkB* transcripts in dental follicle correlates with nerve fiber outgrowth in the dental sac (Byers *et al.*, 1990; Mitsiadis *et al.*, 1993a; Luukko *et al.*, submitted). Similarly, the onset of NGF, gp75^{NTFR}, *trkB*, and *trkC* synthesis in pulpal fibroblasts coincides with the arrival of sensory and sympathetic neurons in dental papilla (Tsuzuki and Kitamura, 1991). During subsequent development, these nerve fibers form a dense plexus in the sub-odontoblastic region. Selected groups of neurons follow NTFs concentration gradients (Gunderson and Barrett, 1979; Klein *et al.*, 1990; Rawdon, 1991). gp75^{NTFR} and truncated Trk receptors expressed in dental mesenchyme (follicle and papilla) may function to increase local concentrations of NTFs (Chao, 1992), thereby providing a tropic and trophic support for growing axons. Neurite arborization occurring in the sub-odontoblastic layer may be promoted by NTFs, since NGF increases arborization of sensory neurons *in vitro* (Yasuda *et al.*, 1990). NTFs may be also responsible for the rich innervation that develops in the mature tooth, according to previ-

ous findings which demonstrate that the density of innervation in an organ can be regulated by NGF (Thoenen *et al.*, 1987). In support of this, the administration of anti-NGF antisera to rat pups (early postnatal stages) reduced the number of axons in the dental papilla (Naffel *et al.*, 1994).

The distribution of NGF molecules in tooth germs cultured in a medium containing 20% fetal calf serum was identical to that observed *in vivo*. Furthermore, the expression patterns of gp75^{NTFR}, *trkB* and *trkC* transcripts were not altered in tooth germs cultured as explants *in vitro* (Luukko *et al.*, submitted). Taken together, these findings indicate that expression of NTFs, gp75^{NTFR} and Trk receptors in the developing tooth is independent of innervation, and suggest that they may be regulated by local mechanisms (e.g. epithelial-mesenchymal interactions) and/or circulating factors. The latter hypothesis was tested in tooth germs cultured in serum-free medium, in the presence or absence of NGF. In both cases the expression patterns of NGF in dental tissues were disturbed, suggesting that circulating factors may be involved in the regulation of NTFs and their receptors in the developing tooth.

Possible non-neuronal functions for NTFs in dental epithelium

At the cap stage, NGF is expressed in the forming stellate reticulum, which is believed to be essential for the formation of the future tooth crown. NGF is absent from cells of the inner dental epithelium, which express gp75^{NTFR}, suggesting that other NTFs are responsible for the mitogenic response of these cells. However, it is not yet clear if binding of NTFs only to gp75^{NTFR} may trigger some of their biological responses (Heumann, 1994). Biological responsiveness to NTFs necessitates interactions with the high-affinity form of the receptors, which require either the coexpression of both gp75^{NTFR} and one (or more) of the Trk receptors (Hempstead *et al.*, 1991; Verdi *et al.*, 1994), or binding to dimers of Trk receptors (Barbacid *et al.*, 1991; Klein *et al.*, 1991).

During bell stage, gp75^{NTFR} in dental epithelium is restricted to cells committed to the ameloblast fate, indicating a possible role for NTFs in dental cell specifications. The absence of gp75^{NTFR} from epithelial cells of the forming root (Mitsiadis *et al.*, 1992) coincides with their inability to synthesize enamel matrix proteins. Furthermore, gp75^{NTFR} expression in cells of the inner dental epithelium follows their proliferative apical direction. NGF is also found in inner dental epithelium. Embryonic stem cells from various tissues express gp75^{NTFR} (Yan and Johnson, 1988), and NTFs are able to trigger and support cell division (Burstin and Greene, 1982; Levi-Montalcini, 1987; Cattaneo and McKay, 1990; Chao, 1992). Together these findings suggest that NGF, alone or in combination with other NTFs, may have a mitogenic function in dental epithelium. Recently, we showed TrkA expression in proliferative cells lacking gp75^{NTFR} (cells of the stratum intermedium and stellate reticulum) (Mitsiadis, 1993; Mitsiadis *et al.*, 1993b; T. Mitsiadis, B.B. Rudkin and D. Martin-Zanca, unpublished results), suggesting that TrkA alone may mediate the proliferative effects of NGF.

Fig. 3. Localization of *trkB* and *trkC* mRNA by *in situ* hybridization in embryonic and post-natal rat tooth germs (E15-PN4). Bright-field (A,C,E,G, and I) and the corresponding dark-field (B,D,F,H, and J) illuminations. (A and B) In the bud staged tooth (E15), *trkB*.T1 transcripts are detected in both dental epithelium (*de*) and condensed mesenchyme (*cm*). (C and D) At the early bell stage (E19), *trkB* transcripts are scarcely expressed in outer dental epithelium (*ode*), cervical loops (thick arrow), and in the cervical part of the dental papilla (*p*, thin arrows). Intense signal is detected in the mesenchyme lining the dental lamina and part of the outer dental epithelium. (E and F) During cytodifferentiation (PN4), moderate *trkB*.T1 hybridization signal is detected in dental papilla mesenchyme (*p*) of the cervical loop region (arrows). The signal observed in ameloblasts (*a*) and odontoblasts (*o*) is an artifact. (G and H) At PN1, *trkC* transcripts are weakly expressed in the dental papilla (*p*) of the developing tooth germ. (I and J) In a PN4 tooth germ, strong *trkC* mRNA hybridization signal is detected in the cuspal areas of dental papilla mesenchyme (*p*), but not in odontoblasts (*o*). The signal observed in ameloblasts (*a*) is artifactual. *Mc*, Meckel's cartilage; *eo*, enamel organ; *d*, dentin; *pa*, preameloblasts. Scale bar, 200 μ m.

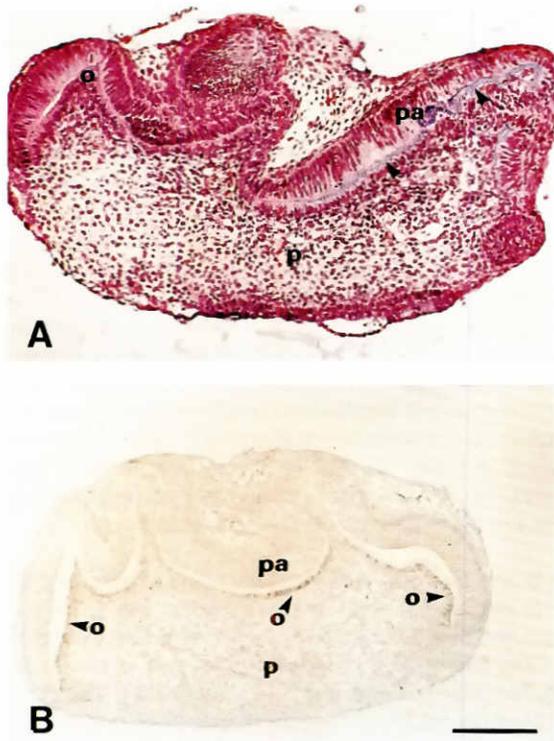


Fig. 4. Effect of NGF on morphogenesis of cultured in a serum free medium E16 tooth explants. (A) Addition of NGF (100 ng/ml) in the medium had no detectable effects on tooth morphogenesis after 5 days of culture. Cytodifferentiation and pre-dentin (arrowheads) deposition are not altered, and cusps develop normally. (B) Localization of NGF-like immunoreactivity in a tooth germ cultured for 5 days in a serum free medium supplemented with 100 ng/ml of NGF. Reactivity is restricted in odontoblasts (o, arrowheads). o, odontoblasts; pa, preameloblasts; p, dental papilla. Scale bar, 100 μ m.

Functional ameloblasts express NGF but not gp75^{NTFR}. The absence of gp75^{NTFR} from ameloblasts does not mean that NGF is not active in these cells. TrkA is found in functional ameloblasts (Mitsiadis, 1993; Mitsiadis *et al.*, 1993b; T. Mitsiadis, B.B. Rudkin, and D. Martin-Zanca, unpublished results), suggesting that NGF could exert its biological functions only through the TrkA in the matured ameloblasts. At the advanced bell stage, NGF immunoreactivity is observed in nerve-like structures localized to the layer of the ameloblasts, suggesting that the ameloblasts may receive a direct neural supply.

Possible non-neuronal functions for NTFs in dental mesenchyme

During the bud and early cap stages, NGF, gp75^{NTFR}, and trkB are detected in the dental mesenchyme. Adhesion functions have been proposed for gp75^{NTFR} and NGF during the growing of axons (Johnson *et al.*, 1988; Chao, 1992). Furthermore, it has been suggested that trkB may also have a role in adhesive interactions, since its amino acid sequence indicates several putative binding sites in the extracellular domain (Schneider and Schweiger, 1991). These data suggest that NTFs and their receptors may play a role in the condensation of the neural crest-derived mesenchyme.

During the presecretory stage, preodontoblasts undergo differentiation into odontoblasts. Induction of NGF, gp75^{NTFR}, and TrkA (Mitsiadis 1993; Mitsiadis *et al.*, 1993b) expression in preodontoblasts correlates with the onset of odontoblast differentiation. Previous data showed that appearance of NGF receptors in neural crest cells coincides with the acquisition of a particular phenotype (Bernd, 1985). If NTFs trigger odontoblast differentiation, they do so in part through the activation of new gene expression. A number of growth factors and cytoskeletal proteins are induced in differentiating odontoblasts (Kubler *et al.*, 1988; Thesleff *et al.*, 1995). NGF induces transcription of *TGF β 1* through a specific promoter element in PC12 cells (Kim *et al.*, 1994), suggesting that activation of *TGF β 1* expression in odontoblasts (Thesleff and Vaahtokari, 1992) is one of the responses of these cells to NGF. These findings illustrate a close correlation between the

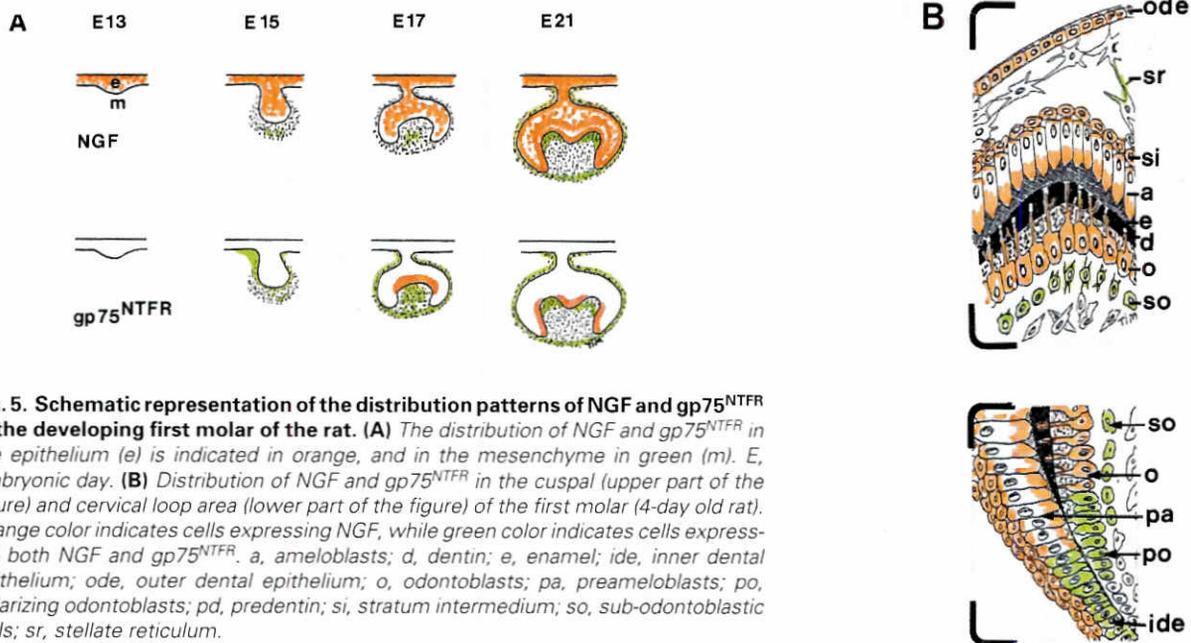


Fig. 5. Schematic representation of the distribution patterns of NGF and gp75^{NTFR} in the developing first molar of the rat. (A) The distribution of NGF and gp75^{NTFR} in the epithelium (e) is indicated in orange, and in the mesenchyme in green (m). E, embryonic day. (B) Distribution of NGF and gp75^{NTFR} in the cuspal (upper part of the figure) and cervical loop area (lower part of the figure) of the first molar (4-day old rat). Orange color indicates cells expressing NGF, while green color indicates cells expressing both NGF and gp75^{NTFR}. a, ameloblasts; d, dentin; e, enamel; ide, inner dental epithelium; ode, outer dental epithelium; o, odontoblasts; pa, preameloblasts; po, polarizing odontoblasts; pd, pre-dentin; si, stratum intermedium; so, sub-odontoblastic cells; sr, stellate reticulum.

appearance of NGF and the differentiation process, and support the notion that NTFs may be implicated in the cascade of regulatory signals which mediate odontoblast differentiation.

Mode of action of NGF in dental tissues

Differentiating odontoblasts and preameloblasts synthesize and secrete NGF molecules (Mitsiadis *et al.*, 1993a), which could then bind to gp75^{NTFR} and TrkA supporting an autocrine mode of action of NGF in these cells. In contrast, dental cells of other layers never co-express NGF and gp75^{NTFR}. This suggests that NGF may affect these cells in a paracrine manner. Paracrine functions for NGF have been reported in human epidermis (Yaar *et al.*, 1991). In dental cells which lack the gp75^{NTFR} it is conceivable that TrkA mediates the effects of NGF (Barbacid *et al.*, 1991; Klein *et al.*, 1991). In this regard gp75^{NTFR} knock-out animals exhibited no obvious defects in organs expressing NGF (heart, lung, and kidney) (Lee *et al.*, 1992; reviewed by Snider, 1994). The lack of any major effect of gp75^{NTFR} knock-out is probably due to compensation by other members of the gp75^{NTFR} family (e.g. OX40, CD30 and CD40), as well as action of other neurotrophic receptor molecules (e.g. TrkA) (for reviews see Chao, 1992; Snider, 1994). Recently, knock-out animals for NTFs and Trk receptors have been generated, and minimal phenotypic changes have been reported (reviewed by Snider, 1994). These animals should afford a useful model for elucidating the potential roles of NTFs in odontogenesis.

In conclusion, our data suggest neuronal and non-neuronal roles for NTFs during odontogenesis. The effects of NTFs could depend on the target cell type and its developmental stage, as well as on other environmental signals. It can be postulated that NTFs play a normal regulatory role in both mesenchymal and epithelial dental cells. Expression of gp75^{NTFR} in cells of the inner dental epithelium may indicate a role in proliferation or phenotypic expression of these cells, while expression in preodontoblasts may suggest the involvement of NTFs in odontoblast terminal differentiation.

Materials and Methods

Preparation of tissues

For immunohistochemistry, tooth germs from both rat embryos and newborn rats were dissected. Preparation of rat tissues has been already described (Mitsiadis *et al.*, 1992, 1993a).

Immunohistochemistry

Immunofluorescent and immunoperoxidase stainings were performed as earlier (Mitsiadis *et al.*, 1992, 1993a). Polyclonal antibodies against NGF were used (see Mitsiadis *et al.*, 1992). Monoclonal antibodies against rat gp75^{NTFR} (IgG-192; Taniuchi and Johnson, 1985) were from E.M. Johnson Jr. (St. Louis, USA).

In situ hybridization

The sites of *trkB* and *trkC* mRNA expression in rat dental tissues were localized by *in situ* hybridization which was performed according to Luukko *et al.* (submitted). Rat *trkB* probe recognizes all forms of the receptor, while the *trkB.T1* probe recognizes a truncated form of the receptor.

Tooth germ cultures and histological procedures

For the experimental *in vitro* analysis of the function of NGF in the developing tooth germs, day-16 and day-17 (E16-E17) first lower molar germs from Swiss mouse embryos were cultured for several days (5-7) either in the absence or presence of NGF (100 ng/ml), on a semi-solid medium, as previously described (Mark *et al.*, 1990). The explants were fixed in Bouin-Hollande and embedded in paraffin, then sectioned, and finally immunostained with anti-NGF antibodies.

Acknowledgments

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