Focal *c-fos* expression in developing rat molars: correlations with subsequent intradental and epithelial sensory innervation

MARGARET R. BYERS[1]*, KELLY B. MECIFI[1] and MICHAEL J. IADAROLA[2]

[1]*Departments of Anesthesiology, Biological Structure and Endodontics, University of Washington, Seattle and* [2]*Neurobiology and Anesthesia Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, USA*

ABSTRACT The purpose of this study was to analyze the temporal and spatial patterns of expression of the inducible transcription protein Fos and the Fos-related antigens (Fra) in developing rat teeth. Immunoreactivity (IR) for Fos/Fra was analyzed at postnatal ages 1-35 days. A transient gradient of Fos/Fra-IR was found in all the molars in the coronal odontoblasts along sites of dentinogenesis, with numerous cells and intense staining near the pulp horn tip, and fewer cells and less staining in mid-crown and cervical pulp. This gradient was well established in first molars of the one day old rats; it was first seen in second molars in the 2 day old rats; and it was found in third molars at 10 days. The Fos/Fra-IR was transient and faded after a few days. Rat molars have a tilted orientation so that maxillary molar crown cusps point in a posterior direction and mandibular crowns in the anterior direction. In each set of molars dentinogenesis was initiated along the side of each pulp horn closest to the gingival surface, i.e. anterior for maxillary crowns and posterior for mandibular crowns; and the Fos/Fra-IR first appeared next to those asymmetric sites. As the wave of dentinogenesis spread around the crown, it was accompanied by odontoblastic expression of Fos/Fra-IR that had decreasing intensity in mid- and cervical crown. Molar root pulp lacked Fos/Fra-IR, and incisor teeth only had odontoblastic and ameloblastic immunoreactivity in the 1 day old rats. Dentinal innervation developed in molars two weeks after the transient Fos/Fra, and it established a similar gradient that was most dense near the crown tips on anterior sides of maxillary molars and posterior sides of mandibular molars. The non-secretory pre-ameloblast epithelium at the molar enamel-free cusp tips also had prolonged Fos/Fra-IR during crown morphogenesis at the enamel-free cusp tips. Sensory innervation was concentrated there prior to eruption, and junctional epithelium appeared to originate from those cells during eruption. The Fos/Fra-IR in developing rat molar odontoblasts and ameloblasts had distinct sites of prolonged expression during crown morphogenesis that did not appear to involve apoptosis, and that matched later sites of sensory innervation.

KEY WORDS: *odontoblasts, ameloblasts, junctional epithelium, CGRP, Fos*

Introduction

The proto-oncogene *c-fos* is one of the immediate early genes that encode inducible transcription factors that are essential for completion of many signal transduction pathways in eukaryotic cells (Greenberg *et al.*, 1985; Curran and Morgan, 1985; Schilling *et al.*, 1991; Curran *et al.*, 1993). These proteins are synthesized in large amounts during proliferation and differentiation by cells responding to mitogens, cytokines, extracellular matrix or growth factors (Crabtree, 1989); during regeneration (Thompson *et al.*, 1986); and in mature cells that are shifting to a different functional state, for example dorsal horn neurons responding to afferent nociceptive signals (Hunt *et al.*, 1987). Many developing or mature cells that are initiating programmed cell death (apoptosis) also express *c-fos* (Buttyan *et al.*, 1988; Fisher *et al.*, 1991; Colombel *et al.*, 1992; Marti *et al.*, 1994); but apoptosis does not necessarily involve the *c-fos* gene (Martin *et al.*, 1992; Dragunow *et al.*, 1993).

The Fos proteins form heterodimers with proteins of another proto-oncogene, *jun*, to become the activating promoter AP-1 transcription factor. These proteins have rapidly-induced and short-lived mRNAs; they can be visualized within 1 hour in nuclei of affected cells by immunocytochemistry or by Fos-LacZ enzyme activity; and in most cases their expression fades within a few hours

Abbreviations used in this paper: CGRP, calcitonin gene-related peptide; fos, proto-oncogene *c-fos*; Fos/Fra, Fos protein and Fos related antigens; IR, immunoreactivity; *lacZ*, gene for ß-galactosidase; NGF, nerve growth factor; p75-NGFR, low affinity receptor for NGF; mRNA, messenger ribonucleic acid.

***Address for reprints:** Anesthesiology, Box 356540, University of Washington, Seattle, WA 98195-6540, USA. FAX: 206-685.3079.

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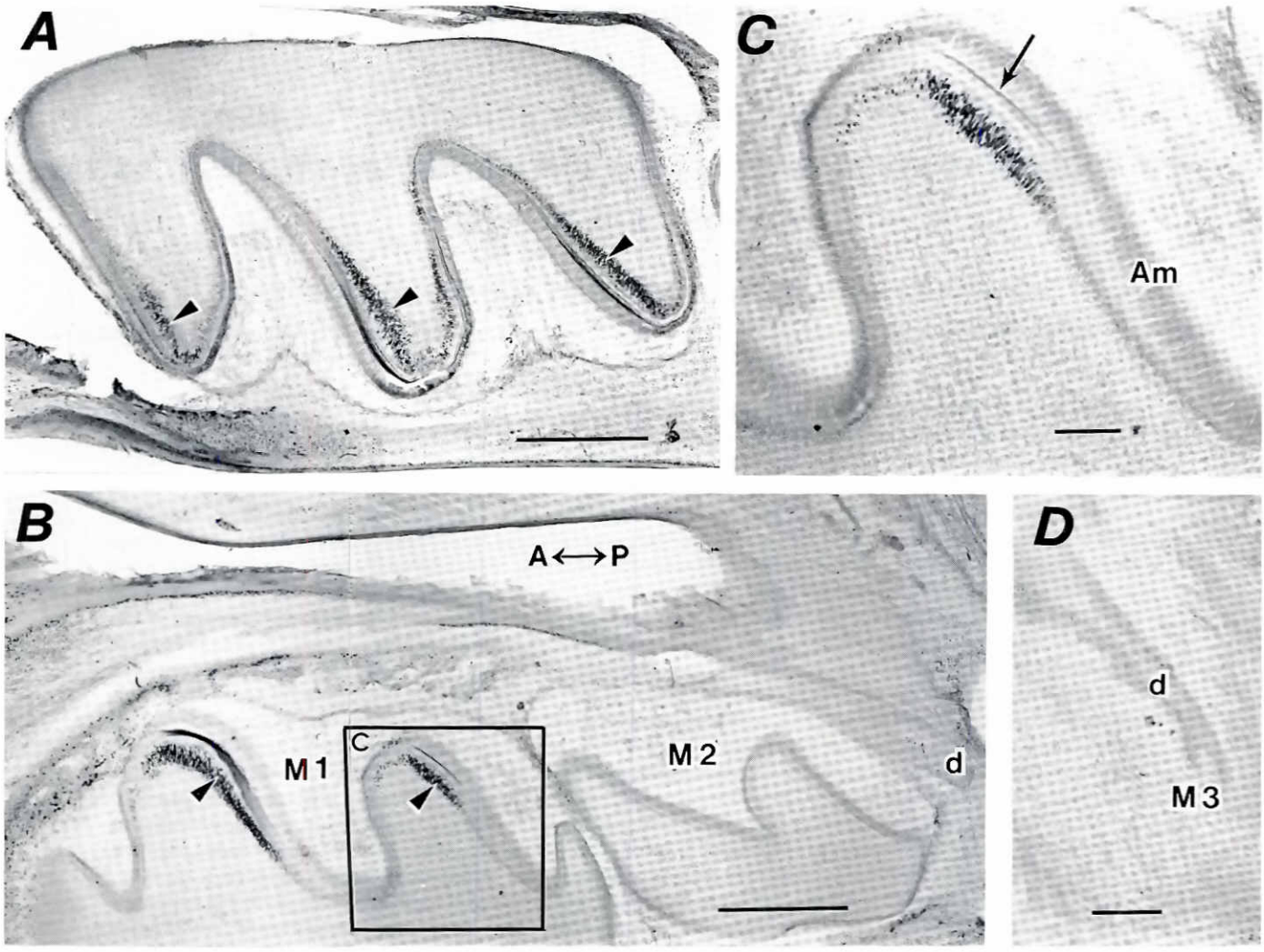

Fig. 1. Asymmetry of pulpal Fos/Fra-IR in undecalcified teeth. *In first molars (M1) of 1-day old rats, many Fos/Fra-IR odontoblasts (arrowheads) are clustered primarily on the anterior side of each maxillary pulp horn* **(A)** *and on the posterior sides of mandibular pulp horns* **(B-C)**; *second molars (M2) are not yet immunoreactive; and third molars (M3) are just starting to form a cap at the dental lamina (d) (Fig. 3B-D). Calcified enamel is indicated (arrow). The ameloblasts (Am) are uniformly columnar on the more developed side of each cusp, whereas the odontoblasts have a shifting morphology with more columnar cells near the cusp tip and fewer, flatter cells in cervical regions. Scales, A,B: 0.5 mm; C,D: 0.1 mm. Anterior, A; Posterior, P.*

(Mueller *et al.*, 1984; Schilling *et al.*, 1991). It has recently been demonstrated that Fos protein mediates transformation of cells during oncogenesis, and that this can happen independently of the cell cycle providing there is an extended period (3 days) of expression (Miao and Curran, 1994). In addition, it has been proposed that continuous expression of *fos* begins many hours or days before apoptosis, and that extended periods of *fos* expression may be "a hallmark of terminal differentiation and a harbinger of death" (Smeyne *et al.*, 1993). The expression of *fos* is therefore not only an indicator of proliferation, differentiation, cellular phenotype conversion, or cell death; but the duration of expression may be diagnostic for the latter event.

Caubet and Bernaudin (1988) have reported that Fos mRNA is expressed in the crown pulp of first molars of newborn mice. During a study of Fos and Fos related antigens (Fra) in apoptotic transition cells of keratinizing epithelia (Fisher *et al.*, 1991), we also analyzed neonatal dental tissue and found distinctive patterns of Fos/Fra immunoreactivity. That work is reported here, and it demonstrates that there is a focal gradient of expression of this transcription factor for extended periods of time at discrete sites during differentiation and maturation of rat molar tissue. Fos/Fra immunoreactivity lasted a few days in coronal odontoblasts and weeks in non-secretory epithelial cells at the enamel-free zones, under conditions that did not appear to include apoptosis. We also report that subsequent development of intradental and epithelial sensory nerve endings followed the same patterns as the Fos/Fra immunoreactivity.

Results

Intradental Fos/Fra in developing rat molar crowns

Opposite asymmetry in maxillary and mandibular molars

Immunoreactivity (IR) for c-Fos/Fra was well developed in the first molars of 1 day old rats, and it was concentrated in odontoblasts along the anterior surfaces of the maxillary first molar pulp horns and along the posterior surfaces of the mandibular first molars (Fig.

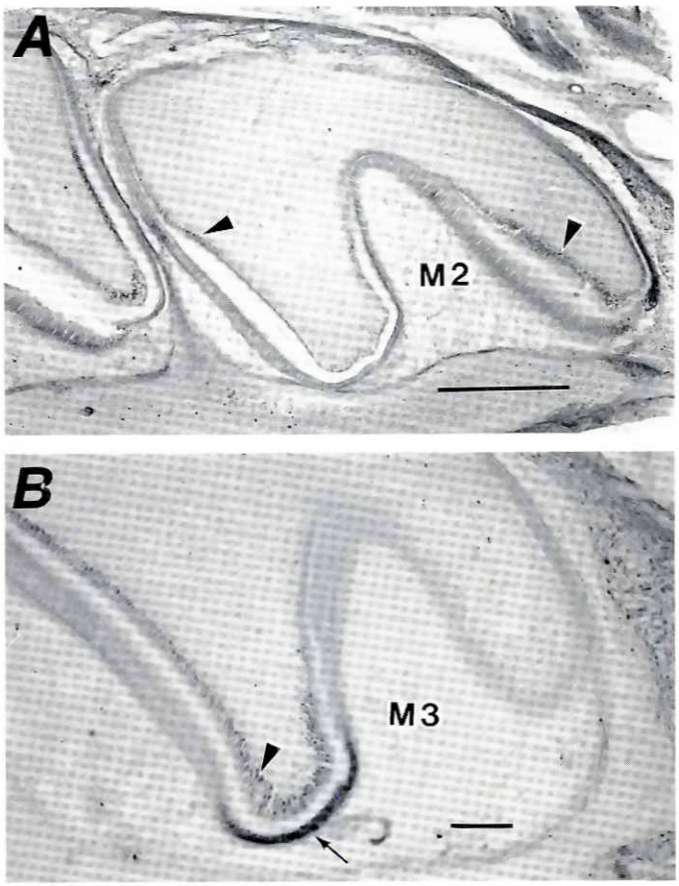

Fig. 2. Shifting Fos-Fra-IR in second and third molars. (A) *Asymmetric Fos/Fra-IR is in the second molar (M2) posterior pulp horn at 4 days, whereas its anterior pulp horn has already lost most of the Fos/Fra-IR at the tip, but has an expression zone in cervical pulp (arrowheads).* **(B)** *The pulpal Fos/Fra-IR was still found in third molars (M3, arrowhead) in 14-day old rats. Initial pre-ameloblast Fos/Fra-IR was also present at the cusp tip (arrow). Scales, A, 0.5 mm; B, 0.1 mm.*

1). These molars are tilted in opposite directions: maxillary crowns towards posterior and mandibular towards the anterior direction. The Fos/Fra-IR odontoblasts are therefore concentrated on the side of the first molar pulp horns closest to the gingival surface. That is also the region where dentinogenesis is most advanced and where amelogenesis begins. In the one day old rats, the second molar does not yet have Fos/Fra-IR in odontoblasts, and the third molar is just beginning to form at the base of the dental lamina and does not have immunoreactivity (Fig. 1). The second molars first had Fos/Fra-IR in two day old rats and the third molars had it at 10 days. In all the molars, the odontoblast expression of Fos/Fra-IR was brief and disappeared from a particular coronal region within a few days (Fig. 2).

Gradients of transient expression in coronal odontoblasts

The Fos/Fra-IR was not found in molars that had begun dentinogenesis but that had not yet started amelogenesis (Fig. 1). It appeared first in midline sections near amelogenic regions, in the most developed pulp horns, and close to the tip of each pulp horn (Figs. 1-3). There was a general correspondence between zones of early amelogenesis and Fos/Fra-IR in odontoblasts; but many regions had amelogenesis that extended further, indicating an earlier onset (Fig. 3A). Although the amelogenesis spread gradually around the crown, the ameloblasts had a uniform columnar appearance all along the most developed sides of the crowns at 1 day (Figs. 1-3). By 4 days after birth, the wave of maturation had spread to include all sides of the first and second molar crowns, and Fos/Fra-IR was now reduced near pulp horn tips in midline regions (Fig. 3B), but still strongly expressed in pulp horns in more lateral or medial regions of the same tooth (Fig. 3C).

Molar roots and incisor teeth

Odontoblasts in developing molar roots or along interradicular dentin did not have transient expression of Fos/Fra-IR (Fig. 4). Instead, the immunoreactivity was present in cementoblasts and

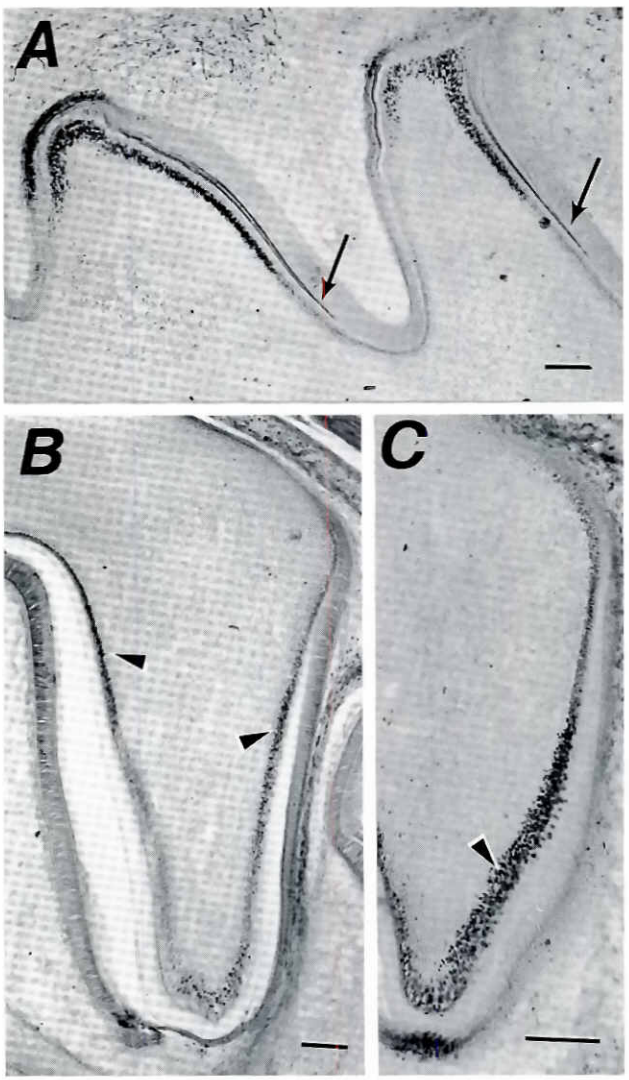

Fig. 3. Shifting Fos/Fra-IR in first molar. *In many cases amelogenesis had begun (arrows) prior to onset of Fos/Fra-IR in the odontoblast layer, shown here in a one-day old mandibular first molar* **(A)**. *By 4 days the Fos/Fra-IR zone had shifted away from the pulp horn tip towards cervical regions in some cases* (**B**, *arrowheads); and in others it had just spread to include the posterior side of the maxillary first molar* (**C**, *arrowhead). Scales, 0.1 mm.*

nearby cells of periodontal ligament along the outer surfaces of the developing roots. Incisor teeth of 1 day old rats had Fos/Fra-IR in developing odontoblasts and ameloblasts along the labial side of the teeth, but there was no intradental Fos/Fra-IR on the palatal or lingual sides (Fig. 5). Those surfaces are covered by cementum instead of enamel, and they had Fos/Fra-IR in cementoblasts and nearby fibroblasts similar to the molar roots. Incisor teeth of all the other ages examined here (2-35 days) had little or no detectable Fos/Fra-IR (Fig. 5D), despite strong immunoreactivity in developing molars, bone, or epithelia.

Asymmetry of developing sensory innervation in molar dentin

When sensory nerve fibers enter first and second molar coronal dentin at 2-3 weeks, they select the anterior surfaces of maxillary molar crowns and the posterior surfaces of mandibular crowns for terminal arborization (Fig. 6). This preferential innervation of one side of each pulp horn is shown here for first and second molars in a 4 week old rat immunoreacted for the neuropeptide CGRP; and the pattern mimics the gradients of Fos/Fra-IR expression in the developing odontoblasts several weeks earlier. This pattern confirms earlier observations about innervation asymmetry when radioactive labeled trigeminal nerve endings were mapped in dentin that also had growth lines showing the time of origin of different dentin regions (Byers, 1980, 1984; Byers *et al.*, 1982). That asymmetric orientation persisted in rat teeth so long as reparative dentin formation had not destroyed the coronal primary dentin (Fig. 7).

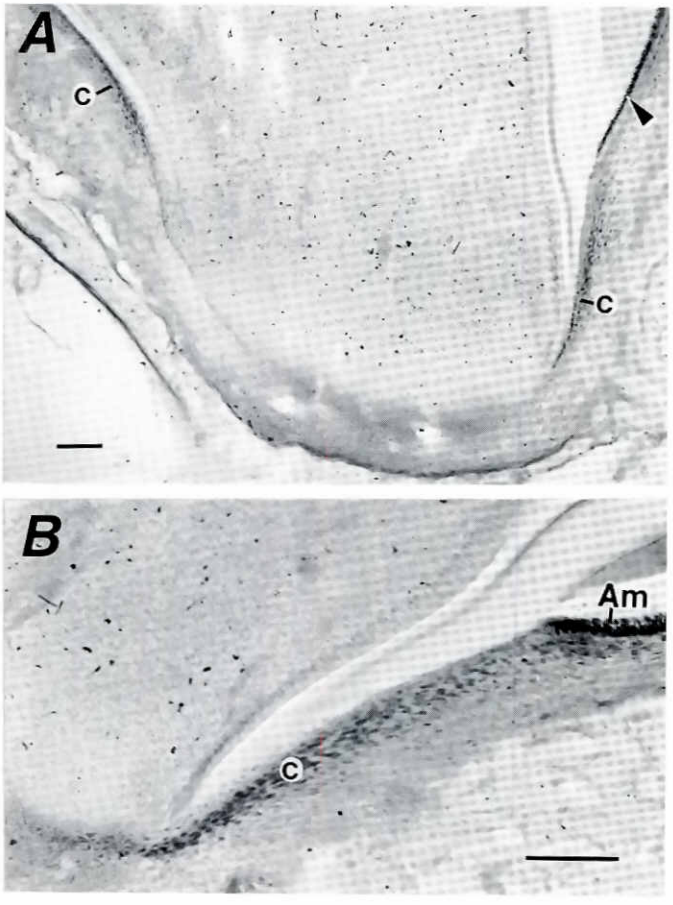

Fig. 4. Odontoblasts in root pulp did not have Fos/Fra-IR. *Instead immunoreactive cementoblasts (c) and associated cells of periodontal ligament were found along the periodontal root surfaces. Intense Fos/Fra-IR ameloblasts (Am, arrowhead) were present in these 2 week old first molars. No counterstain. Scales, 0.1 mm.*

Expression of Fos/Fra in ameloblasts

Intense immunoreactivity for Fos/Fra-IR was found in non-secretory ameloblast epithelium at the enamel-free tip of each molar cusp. It was present soon after onset of dentinogenesis (Figs. 2B, 3C, 8A) and continued through crown morphogenesis (Fig. 8B). Those areas came in contact with the submucosal tissue after the bell stage when the enamel organ fragmented, and they retained their intense Fos/Fra-IR all the way up to eruption. Regular ameloblasts only had Fos/Fra-IR just prior to eruption.

Focal innervation of non-secretory enamel epithelium and initial junctional epithelium

Sensory nerve fibers that were immunoreactive for CGRP were found associated with the Fos/Fra-IR non-secretory ameloblast epithelium at the enamel-free zones (Fig. 8C-D). As the molar cusps erupted, those cells and their associated nerves were displaced to the side of each cusp and appeared to form the new junctional (attachment) epithelium (Fig. 8C-D). That epithelium was more stratified than the remaining enamel epithelium; and it was innervated (Fig. 8E-G). It also had reduced its Fos/Fra-IR compared to adjacent apoptotic transition cells of the eruption wound and of keratinized epithelium with intense Fos/Fra-IR (Fig. 8H).

Discussion

The patterns of Fos/Fra expression that we found in developing rat molar crown pulp had asymmetric gradients that matched the termination patterns of sensory nerves arriving to innervate the odontoblast layer and adjacent dentin several weeks later. The Fos/Fra-IR and innervation were concentrated on the anterior sides of maxillary molar and posterior sides of mandibular crown pulp, with the greatest intensity near the tip and decreasing towards cervical crown regions. Many other features of rodent molar structure have been found to have a similar gradient that is most intense for the sides of each unerupted molar closest to the gingival surface. These include dentin matrix components such as gamma-carboxyglutamic acid-containing proteins (Finkelman and Butler, 1985), dentin phosphoprotein (MacDougall *et al.*, 1985), osteonectin (Holland *et al.*, 1987), type I collagen and dentin sialoprotein (Bronckers *et al.*, 1993); and other extracellular matrix molecules and growth factors (Ruch, 1985; De Vries *et al.*, 1987; Slavkin, 1991; Thesleff and Vaahtokari, 1992). The capillary plexus in the rat molar crown develops with the same asymmetry described above during the early bell stage; and it is immunoreactive for laminin in rat (Fristad, *et al.*, 1994) and for p75-NGFR in cat teeth (Fried and Risling, 1991) — two molecules that are important for the guidance of growing axons. The pulpal vasculature may therefore provide an important framework for developing nerve fibers that begin dentin innervation just prior to tooth eruption (Byers, 1980; Fristad *et al.*, 1994). Other histologic features of mammalian teeth that correlate with asymmetric innervation gradients are wide predentin and a columnar odontoblast form (Corpron and Avery, 1973; Byers and Dong, 1983; Byers, 1984).

An important orienting factor for the developing nerve fibers must be the intense synthesis of NGF and p75-NGFR by the

fibroblasts beneath the most mature odontoblasts and innervated dentin (Byers *et al.*, 1990, 1992b; Mitsaidis *et al.*, 1992; Naftel *et al.*, 1992). When dentin is labeled with radioactive growth lines to show the times of synthesis and therefore the dentin age, sensory innervation is most intense at the oldest sites on the anterior sides of maxillary molars and the posterior sides of mandibular molars (Fig. 7) (Byers, 1980, 1984; Byers *et al.*, 1982). Those patterns in rat molars are maintained until reparative dentin is made and the adjacent pulp halts the production of NGF and p75-NGFR (Byers *et al.*, 1990; Byers, 1994). The use of silver stains (Corpron and Avery, 1973) and neural immuno-cytochemical markers have found similar asymmetric innervation patterns in rodent molars (Maeda *et al.*, 1987; Byers *et al.*, 1990; Fristad *et al.*, 1994). The importance of the pulpal expression of NGF is indicated by the reduced innervation of molars in rats that have been autoimmunized against NGF (Naftel *et al.*, 1994). Finally, the Fos/Fra-IR in developing rat incisors was different from the molars, and those teeth have only meager sensory innervation (Byers, 1984). The brief coincidence of *c-fos* expression in developing molar odontoblasts next to NGF-producing pulp fibroblasts suggests a specific response to NGF (Curran and Morgan, 1985; Greenberg *et al.*, 1985); but further work is needed to understand odontoblastic *c-fos* expression.

We also found an extended period of Fos/Fra expression in the non-secretory ameloblast epithelium at the tips of each cusp where enamel fails to form (Figs. 1C, 2B, 3C, 8A,B). Those cells also received sensory innervation as soon as the outer enamel epithelium degenerated and the cusps came in contact with the gingival mesenchyme (Fig. 8C-D). The cusp tip ameloblasts did not appear to be undergoing apoptosis during the 2 week period before eruption, since their nuclei were not fragmented. Those cells are derived from the inner enamel epithelium; and they are equivalent to the reduced enamel epithelium of human teeth (Schroeder, 1986). They retain immunoreactivity for protein gene product 9.5 (Fristad *et al.*, 1994) and transforming growth factor ß1 (Vaahtokari *et al.*, 1991) after the rest of the enamel epithelial cells have lost those traits. Analysis of erupting first and second molars at 14-21 days and erupting third molars at 4-5 weeks suggested that the innervated non-secretory epithelial cells at the cusp tips might become the innervated junctional epithelium when those cusps erupt. Serial sections show the innervated epithelial cells shifting to the sides of the cusps. The new collar of innervated junctional epithelium around the erupting molar cusps also had moderate Fos/Fra-IR, while enamel epithelium that persisted along the deeper, still unerupted regions of the crown did not (Fig. 8E-G). Previous studies have compared the innervation of new junctional epithelium to adjacent uninnervated enamel epithelium (Nagata *et al.*, 1992; Fristad *et al.*, 1994). The present study suggests that junctional epithelium in rats may develop from the special non-secretory epithelial cells that line the cusp tips, that continuously express Fos/Fra for several weeks without any apparant apoptosis, and that are innervated prior to eruption. This would be consistent with earlier analyses of the development of junctional epithelium in humans (Schroeder, 1986).

Fos-lacZ expression in transgenic mice can be prolonged in some apoptotic developing tissues such as ovarian follicles or corpus luteum. That observation lead to the suggestion that prolonged *fos* expression signifies impending cell death (Smeyne *et al.*, 1993) even though there was also extended *fos* expression in the developing dental follicle, and other tissues that are not likely to be dying (see also Ferguson, 1993). In the present study, neither

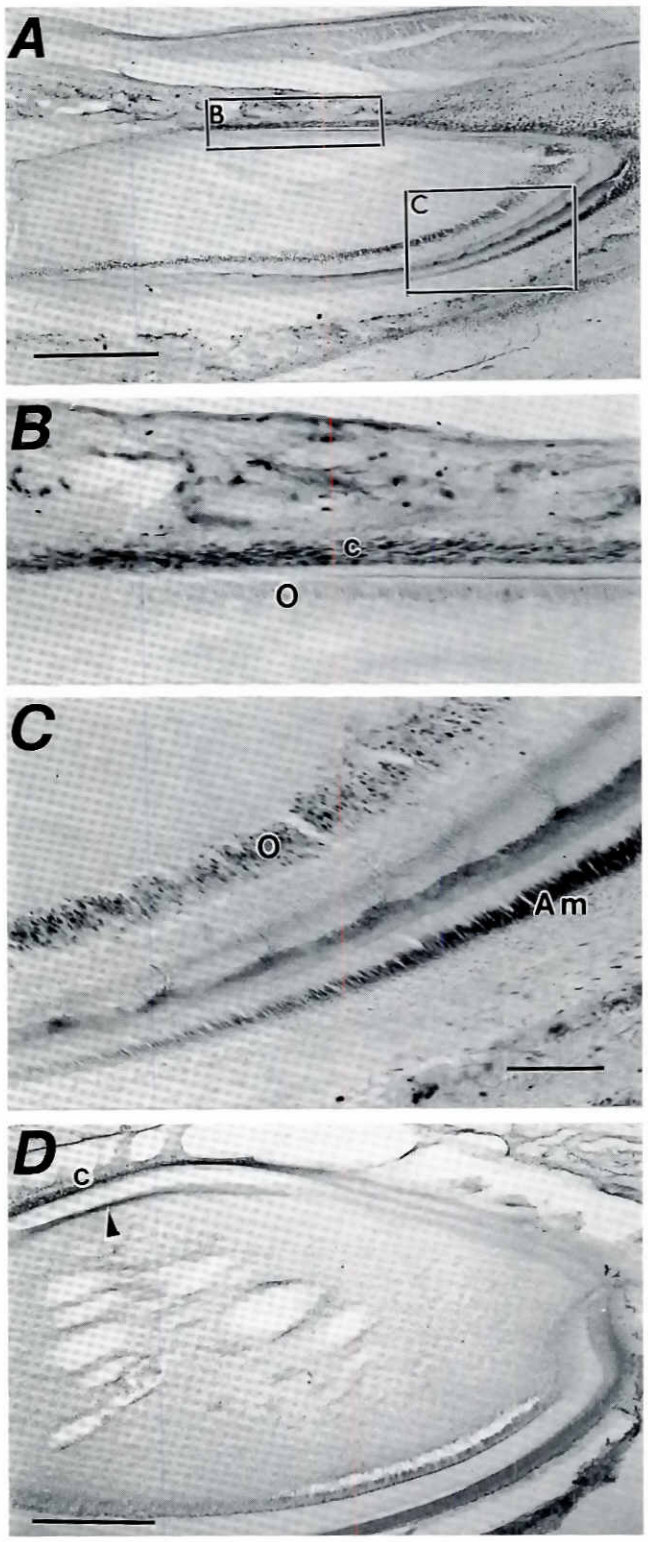

Fig. 5. Rat incisor. (A-C) *Incisor of a 1 day old rat had Fos/Fra-IR odontoblasts (O) and ameloblasts (Am) on the labial side* **(A,C)**; *but only cementoblasts (c) were labeled on the lingual side* **(B). (D)** *Very few Fos/IR cells were found in incisors of rats aged 2-35 days. Here there are a few labeled cementoblasts (c) and odontoblasts (arrowhead) at the base of an incisor of a 4 week old rat. Scales, A-C: 0.1 mm; D: 0.5 mm.*

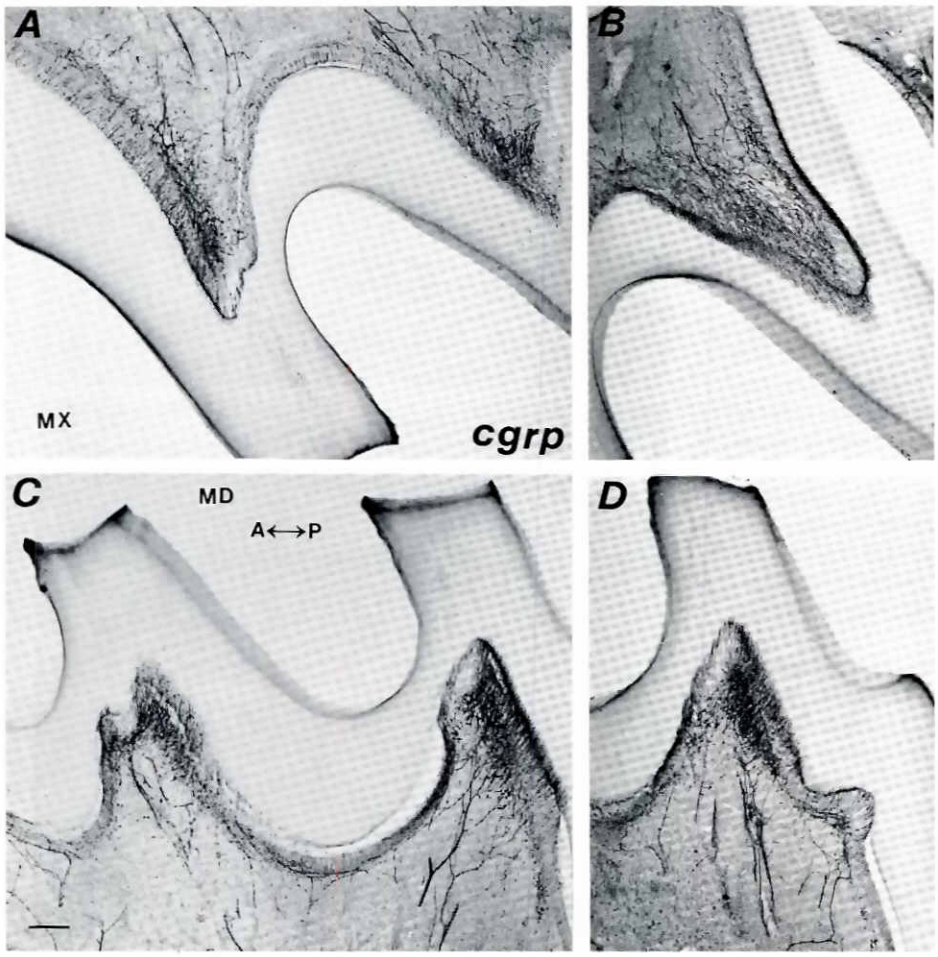

Fig. 6. Asymmetric sensory innervation. *CGRP-IR nerve fibers had preferentially entered dentin on the anterior side of maxillary molars M1* **(A)** *and M2* **(B)** *and the posterior sides of mandibular molars M1* **(C)** *and M2* **(D)** *at about 2 weeks, as seen in this 4 week old rat. MX, maxillary; MD, Mandibular; Anterior, A; Posterior, P. Scale, 0.1 mm.*

the developing odontoblasts nor the non-amelogenic epithelial cells at the cusp tips appeared to be dying, because their nuclei did not become fragmented; whereas fragmented Fos/Fra-IR nuclei were seen in cells along the wound edge during eruption (Fig. 8H). All the coronal odontoblasts in the developing rat molars studied here had a period of several days expression of Fos/Fra. They could not have been doing so as part of an apoptotic program, because they are needed to produce and maintain primary coronal dentin for the life of the tooth. The non-amelogenic epithelial cells had a longer period of continuous Fos/Fra-IR of about 2 weeks prior to eruption (Fig. 8b), and they also maintain production of transforming growth factor ß1 (Vaahtokari *et al.*, 1991) and protein gene product 9.5 (Fristad *et al.*, 1994). Thus, the two populations of cells studied here had prolonged expression of Fos/Fra, unrelated to any detectable apoptosis.

Two other types of experiments provide some hints that there may be correlations between the asymmetric expression of *fos* by developing dental tissues, their subsequent innervation, and odontogenesis. First, when molars are extracted and then re-

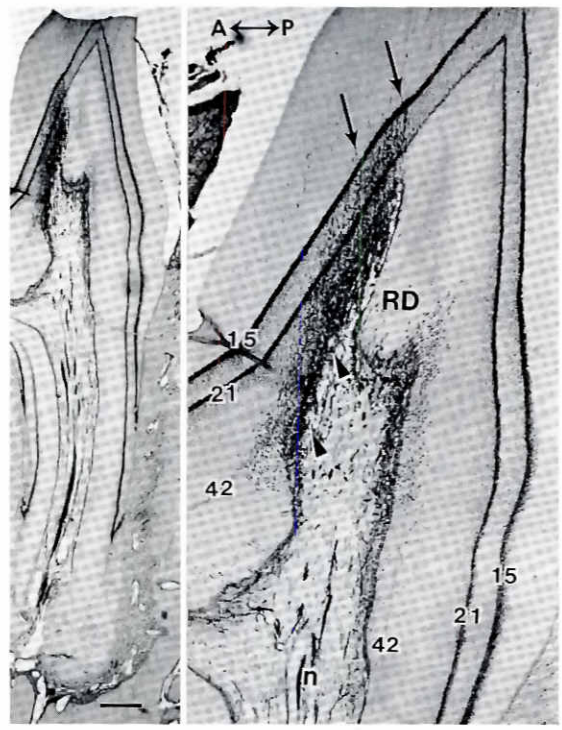

Fig. 7. Nerve position during molar aging. *Growth lines were made in the dentin of this molar by injecting 3H-proline into the tail vein at 15 d, 21 d and 42 d of age. The trigeminal innervation was labeled for autoradiographic mapping when the rat was 106 d old. Those nerve fibers (n) still terminated in the oldest dentin (arrows) on the anterior side of this maxillary molar (for methods see Byers, 1980; Byers et al., 1982). Anterior, A; Posterior, P; RD, reparative dentin. Scale, 0.1mm.*

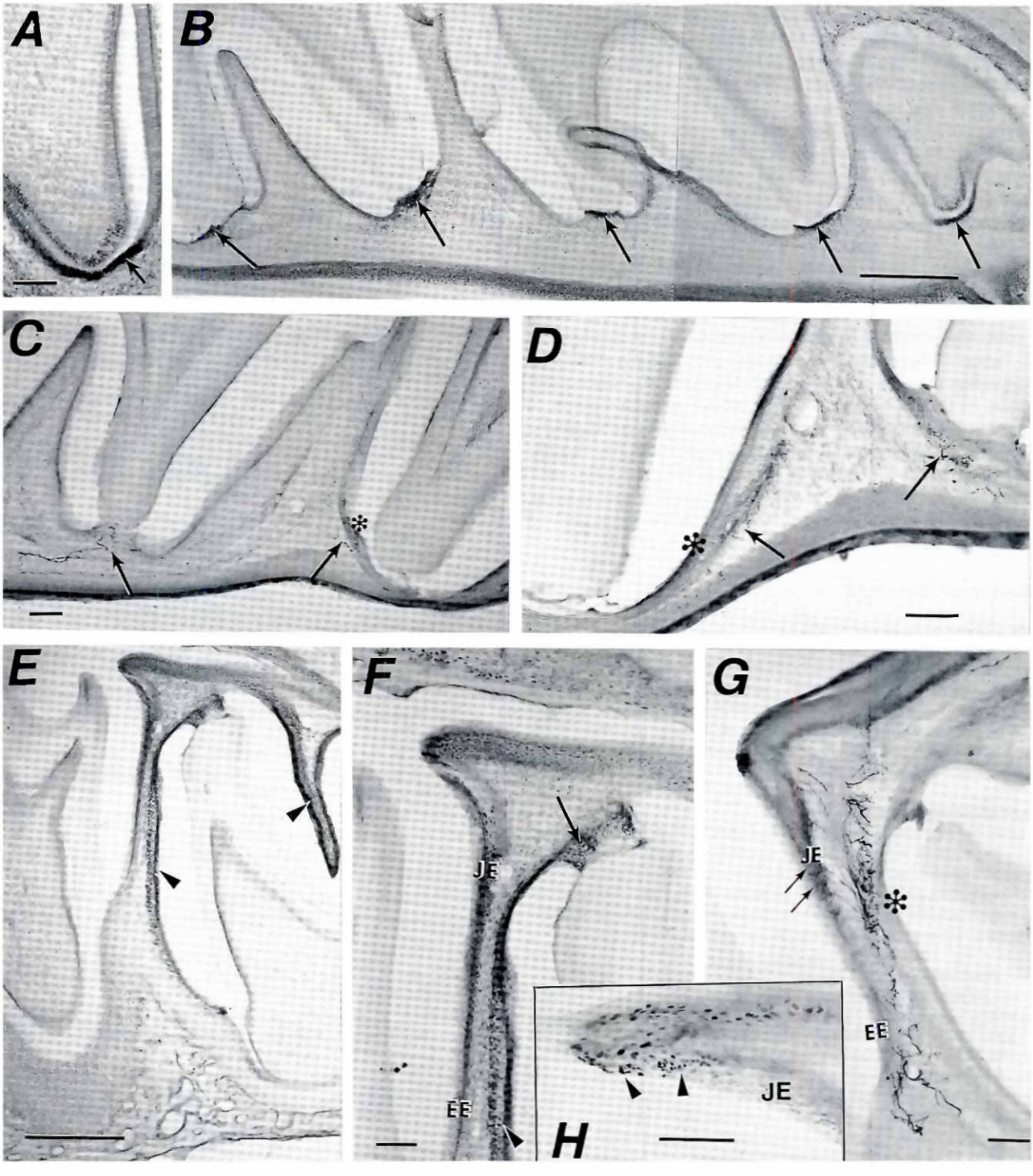

Fig. 8. Ameloblasts. *Some cells in the pre-ameloblast layer at the tip of each molar cusp have Fos/Fra-IR in the bell stage* **(A)**; *those cells are concentrated in the non-secretory zone where enamel is sparse or absent. They continue expressing Fos/Fra-IR (arrows) for several weeks as seen in the 3 molars of a two-week old rat* **(B)**. *Those cells acquire sensory CGRP-IR innervation* (**C,D**, *arrows) once the enamel organ fragments. When the first molar begins to break through the epithelium during eruption in a two-week old rat, the innervation zone moves to the side (asterisk)* **(C-D)**. *An erupted second molar and erupting third molar are shown from a four-week old rat* **(E-H)**. *The c-Fos/Fra-IR is intense in cells at the gingival wound* **(E,F,H)**, *but less intense at the thickened developing junctional epithelium (JE) and weak in the adjacent enamel epithelium (EE) of the erupted tooth. Prior to eruption most of the reduced enamel epithelium has Fos/Fra-IR (8EF, arrowheads). The JE is innervated as shown by immunoreactivity for the p75-NGFR but the EE is not* **(G)**. *The nerve fibers innervate both the new JE of the 2nd molar (small arrows) and the shifting reduced epithelium for the third molar (asterisk). A higher magnification of the epithelial wound at an eruption site shows the intense Fos/Fra-IR of fragmented apoptotic nuclei (arrowheads) compared to the JE* **(H)**. *Scales, A,C-H: 0.1 mm; B, 0.5 mm.*

planted in young rats, the teeth that become reinnervated with CGRP-IR nerve fibers retained normal pulp and dentin structure, whereas those that have little or no reinnervation had become filled with bone (Kvinnsland *et al.*, 1991; Byers *et al.*, 1992a). The CGRP-IR nerve fibers may therefore help to maintain the differentiated pulp and dentin structure and prevent conversion to bone. Second, knockout mice that have a null mutation for the *c-fos* proto-oncogene have malformed, root-less molar crowns that cannot erupt because of excessive overlying bone (Johnson *et al.*, 1992); and their molars appear to have amelogenesis at the tips of the cusps, rather than an enamel-free zone (Fig. 2C, Wang *et al.*, 1992). The absence of *fos* expression in the mutant mice prevents some of the focal specializations of crown formation, as well as allowing overexpression of bone. Those are two events that would affect nerve fibers seeking to innervate specific sites in developing dental tissue. These studies suggest interactions between morphogenesis of special structures in teeth, coronal and epithelial distribution of CGRP-IR sensory innervation, and prevention of bone formation in pulpal or subgingival tissues. Further work is now needed to fully understand the intrapulpal gradients of Fos/Fra-IR, the special prolonged Fos/Fra-IR in non-amelogenic epithelial cells, the relationship of that special epithelium to the junctional epithelium, and the interactions of sensory nerve fibers with all those developing tissues

Materials and Methods

Animals

For each postnatal age, we examined the following numbers of jaws/rats: 1 d (9/5); 2 d (2/1); 4 d (5/2); 6-7 d (5/3); 9-10 d (4/2); 14 d (6/3); 21 d (2/11); 4-5 weeks (3/2). All animals were deeply anesthetized with intraperitoneal Equithesin (0.97% pentobarbital, 4.25% chloral hydrate) and fixed by perfusion with 4% formaldehyde (either with or without 0.2% picric acid added) in 0.1 M phosphate buffer and then subsequent immersion fixation overnight in the same solution.

Tissue processing and decalcification

We used the rapid formic acid technique (Kristensen, 1948) with daily changes for 5-7 days. An initial comparison of undecalcified tissues in 1d neonates and decalcified jaws found no difference in the intensity of the immuno-reactivity (compare Fig. 1, undecalcified, with Fig. 3, decalcified). Decalcification periods from one week to several months were used for the 2-35d rats without deleterious effects on the antibody reactions.

Immunocytochemistry

Primary polyclonal anti Fos/Fra was similar to that described previously (Draisci and Iadarola, 1989; Quinn *et al.*, 1989; Noguchi *et al.*, 1990; Fisher *et al.*, 1991; Iadarola and Messersmith, 1994). It was used at 1:2500-1:15,000 for incubation of floating sections 48-60 h at 4°C. Subsequent steps for immunocytochemistry used standard procedures (Kimberly and Byers, 1988; Byers *et al.*, 1990). Immunocytochemistry with polyclonal anti CGRP (Cambridge Research) used previously described methods (Kimberly and Byers, 1988). Chromogen reactions used standard diaminobenzedine reactions. In most cases basophilic counterstain was also used (but omitted for Fig. 4).

Microscopy

All teeth were examined in sagittal sections that included every second or third serial section for Fos/Fra-IR. Photographs were taken of representative midline areas at each developmental stage using a Nikon FXA microscope.

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