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*Original Article*

# Combinatorial expression patterns of the connexins 26, 32, and 43 during development, homeostasis, and regeneration of rat teeth

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**ABSTRACT** **Gap junctions permit the exchange of regulatory molecules between cells and play important roles during organogenesis. The expression pattern of the gap junction proteins connexin 26, 32, and 43 was studied by immunohistochemistry in the developing, adult, and injured rat teeth. Connexins 32 and 43, but not the connexin 26, were detected during the late stages of embryonic tooth development (bell stage). Expression of connexin 32 was predominant in epithelial cells, whereas connexin 43 was more widely distributed and found in both epithelial and mesenchymal cells. During cytodifferentiation (early postnatal stages), both connexin 32 and 43 were expressed in the epithelial-derived ameloblasts, synthesizing and secreting the enamel matrix proteins. In mesenchyme, connexin 32 was observed only in differentiating odontoblasts, while connexin 43 was expressed in both differentiating and functional odontoblasts, which secrete the dentin matrix. In adult rat teeth, connexin 26 and 43 were expressed in the odontoblastic layer at low and high levels, respectively, while connexin 32 was absent from odontoblasts. Electron microscopy showed that connexin 43 was distributed exclusively at sites of contacts between odontoblasts. However, double immunostaining combined with confocal microscopy suggested an occasional overlap between odontoblasts and calcitonin gene-related peptide-positive nerve fibers. Denervation experiments showed that the expression of connexins in dental pulp was independent of innervation, whereas in injured teeth connexin 43 was upregulated in pulpal fibroblasts. Finally, cultured dental epithelial cells expressed both connexin 32 and 43, and connexin 43 was detected in cultured pulp fibroblasts *in vitro*, thus mimicking the *in vivo* distribution pattern of connexins. These results demonstrate that connexins are involved in tooth development and suggest that a given connexin may have distinct roles during odontogenesis and tooth homeostasis.**

KEY WORDS: *gap junction, ameloblast, odontoblast, tooth, nerve fiber*

## Introduction

Cell-cell communications are important for organogenesis, cytodifferentiation and tissue homeostasis. Adjacent cells can directly exchange ions and small regulatory molecules through channels interconnecting their cytoplasms. These channels are called gap junctions (GJs) and are exclusively formed from polypeptides produced by a multigene family divided into an $\alpha$ and a $\beta$ class. The GJs proteins are named connexins (Cx) and differ in their molecular mass (see Kumar and Gilula, 1992). Connexin 43 (Cx43), connexin 32 (Cx32), and connexin 26 (Cx26) are the first isolated and best known Cx, and they are the products of the $\alpha_1$, $\beta_1$, and $\beta_2$ GJ genes, respectively. Cx32 and Cx26 were first isolated from mammalian liver (Kumar and Gilula, 1986; Zhang and Nicholson, 1989), while Cx43 was isolated from heart tissue (Beyer *et al.*, 1987). These Cx have also been detected in other tissues and organs, such as the brain, thyroid gland, endothelial cells and fibroblasts (Beyer *et al.*, 1987, 1989; Guerrier *et al.*, 1995), indicating that their expression patterns are tissue- and cell type-specific. Recently, it has been shown that the developing dental tissues also express Cx43 (Dahl *et al.*, 1995; Kagayama *et al.*, 1995), suggesting that GJs may be important for dental patterning.

The tooth is an organ that develops as a result of sequential and reciprocal interactions between the oral ectoderm and the neural

*Abbreviations used in this paper*: Cx, connexin; GJ, gap junction; CGRP, calcitonin gene-related peptide; EM, electron microscopy; E, embryonic day; PN, postnatal day; Ir, immunoreactivity.

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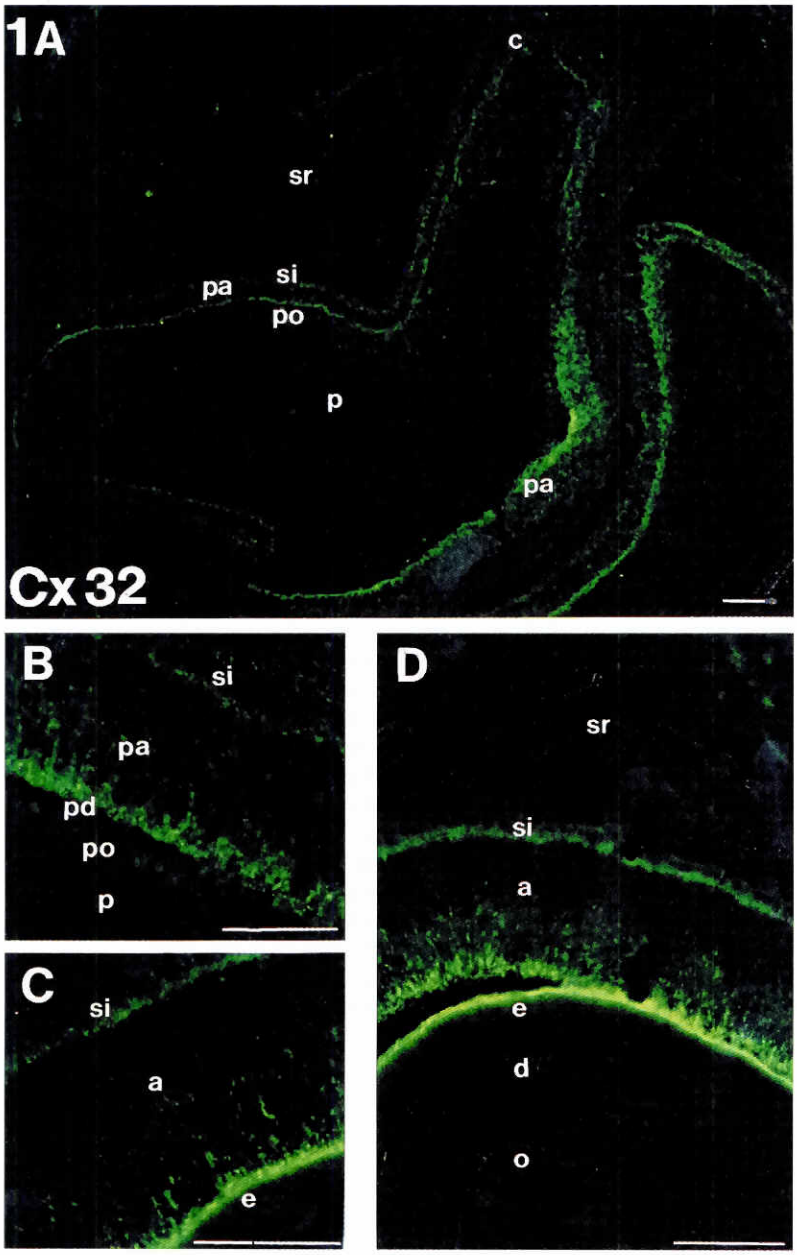


**Fig. 1. Immunohistochemical localization of connexin 32 in the developing molar of the rat. (A)** *Bell staged tooth (E21). Staining is evident in preameloblasts (pa) and cells of the stratum intermedium (si), whereas in preodontoblasts (po) labeling is very faint. Staining is absent from the dental pulp (p) and stellate reticulum (sr).* **(B)** *Higher magnification of the cuspal area (c) of Figure 1A. Note that connexin 32 is distributed in the apical part of preameloblasts.* **(C)** *Higher magnification of the cuspal area of a tooth of a 3 day-old rat. Connexin 32 staining is detected in the apical part of ameloblasts (a) and in cells of the stratum intermedium.* **(D)** *Higher magnification of the cuspal area of a tooth of a 5 day-old rat. The pattern of connexin 32 immunoreactivity persisted in dental epithelium, whereas staining is absent from odontoblasts (o). pd, predentin; e, enamel; d, dentin. Bars, 50 μm.*

crest-derived mesenchyme. The epithelial-derived ameloblasts elaborate the enamel matrix, while the mesenchyme-derived odontoblasts synthesize and secrete the organic components of dentin. Odontoblasts are essential for the transfer of metabolites between pulp and dentin, and form a layer with an epithelial appearance which serves as a protective barrier for the tooth pulp. Furthermore, the close relationship between odontoblasts and terminal nerve fibers in the odontoblastic layer has promoted the hypothesis that the odontoblast process might act as a sensory receptor, coupled to axons within or outside dentinal tubules (see Hildebrand *et al.*, 1995). Although intercellular junctions between odontoblasts have been found earlier by electron microscopy (EM) (Holland, 1976, 1987; Köling and Rask-Andersen, 1981, 1984a,b; Calle *et al.*, 1985), the identification of GJs by EM is very limited as it depends on the appropriate plane and area of the section studied. For these reasons, it has not been possible to study in detail the distribution and developmental regulation of GJs in the dental pulp. Although features suggestive of contacts between odontoblasts and nerve fibers have been described, typical synaptic or electrotonic junctions have not been observed (see Holland, 1980, 1987; Byers, 1984; Närhi, 1985). The pulp-dentin border region contains odontoblasts and terminal nerve axons, often unensheathed by Schwann cell cytoplasm (Holland, 1980; Fried and Risling, 1991; Fried *et al.*, 1992). This makes the identification of cellular processes belonging to particular cell types unreliable, and it is thus very difficult to investigate possible specialized junctions between nerve fibers and odontoblasts on the basis of regular EM. However, this obstacle can be overcome if specific immunohistochemical markers are utilized.

Here we have studied and compared the expression patterns of Cx26, Cx32, and Cx43 in the developing, adult, and injured rat teeth. The possibility of heterologous gap junctional communication between nerve fibers and odontoblasts was examined by double immunostaining using the Cx43 and calcitonin gene-related peptide (CGRP) antibodies.

## Results

### *Immunohistochemical localization of Cx 26, 32, and 43 in the developing, adult and denervated rat teeth*

In the developing molar of the rat (embryonic day 20 to postnatal day 5; E20-PN5), Cx32 immunoreactivity (Ir) was present in the apical part of preameloblasts and ameloblasts, and at lower levels in cells of the stratum intermedium (Fig. 1A-D). A faint Cx32-Ir was observed in differentiating odontoblasts (Fig. 1A and B), but staining was absent from functional odontoblasts and pulpal fibroblasts. Cx43-Ir was detected in both epithelial and mesenchymal dental components (Fig. 2). At embryonic stages, Cx43 was intensely expressed in the oral epithelium overlying the tooth germs (Fig. 2A) and in proliferating cells of the inner dental epithelium and stratum intermedium of the molar tooth germ (Fig. 2B). During cytodifferentiation (PN5), moderate Cx43-Ir was found in the apical part of functional ameloblasts and in functional odontoblasts, whereas the signal was stronger in stratum intermedium (Fig. 2C). In the E21-PN1 incisor, Cx43-Ir was detected in mesenchymal stem cells located in the posterior part of

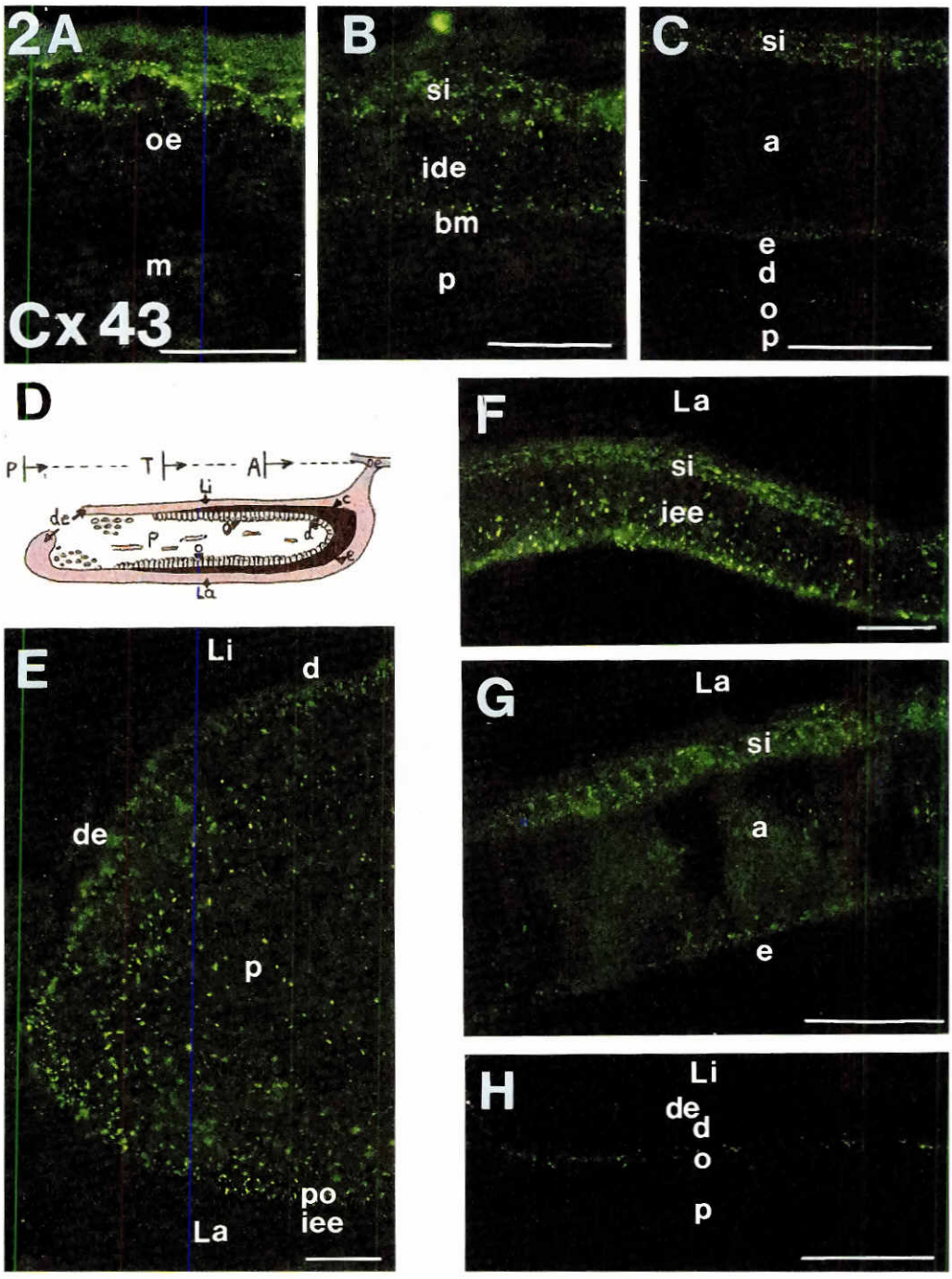


**Fig. 2. Connexin 43 immunoreactivity in the developing molar and incisor of the rat. (A)** *At E21, the oral epithelium (oe) overlying the developing molar exhibits a strong staining, while the mesenchyme (m) is negative.* **(B)** *Higher magnification of the cuspal area of bell staged molar (E20). Staining for connexin 43 is found in the stratum intermedium (si) and inner dental epithelium (ide), but not in pulpal cells (p) contacting the basement membrane (bm).* **(C)** *At postnatal day 3, immunoreactivity is detected in the stratum intermedium, the apical part of ameloblasts (a), and odontoblasts (o).* **(D)** *Schematic representation of a longitudinal section through the incisor of the neonatal rat. Li (pink), lingual side; La (violet), labial side; oe, oral epithelium; de, dental epithelium; p, dental papilla; o, odontoblasts; d, dentin; c, cementum; e, enamel; P, posterior (proliferative) part; T, transition (cytodifferentiation) part; A, anterior (maturation) part.* **(E)** *Higher magnification of the posterior part of an incisor of an E21 rat embryo. Note that the staining is located in proliferating mesenchymal cells, whereas dental epithelial cells are negative.* **(F)** *Higher magnification of the transition part (labial side) of an E21 incisor. Cells of the inner enamel epithelium (preameloblasts, iee) and stratum intermedium express connexin 43.* **(G)** *Higher magnification of the anterior part (labial side) of an incisor of a 3 day-old rat. Staining persists in the stratum intermedium and in the apical part of ameloblasts.* **(H)** *Higher magnification of the transition part (lingual side) of an incisor of a 3 day-old rat. Staining is found in odontoblasts, but not in dental epithelial cells of the lingual side. Bars, 50 μm.*

the incisor (Fig. 2E), proliferating epithelial cells of the labial side (Fig. 2F), and functional ameloblasts located in the anterior part of the incisor (Fig. 2G). No such staining was observed in dental epithelial cells of the lingual side, which are unable to differentiate into ameloblasts, whereas Cx43-Ir was found in functional odontoblasts of both sides (Fig. 2H). Cx26-Ir was difficult to detect in dental tissues at early postnatal stages (data not shown).

In teeth of PN5 rats, the pulps displayed a weak and punctuate Cx43-Ir (Fig. 3A-B). In the odontoblastic layer, Cx43-Ir was more intense compared to the underlying connective tissue. The pattern of Cx43-Ir persisted in ameloblasts, whereas in the stratum intermedium staining was very strong and moderate in the stellate reticulum and the outer dental epithelium (Fig. 3A-B).

In pulps of adult teeth (Fig. 3C-D), Cx43-Ir was mainly detected in the odontoblastic layer, while staining was faint in the pulpal fibroblasts. Cx32-Ir was not observed in the odontoblastic layer of adult pulps, while a very weak Cx26-Ir was occasionally found in this region (data not shown). Intense Cx43-Ir was present in the odontoblastic layer. Often, Cx43-Ir was seen extending into the dentin along the odontoblast processes (Fig. 3D). In the central

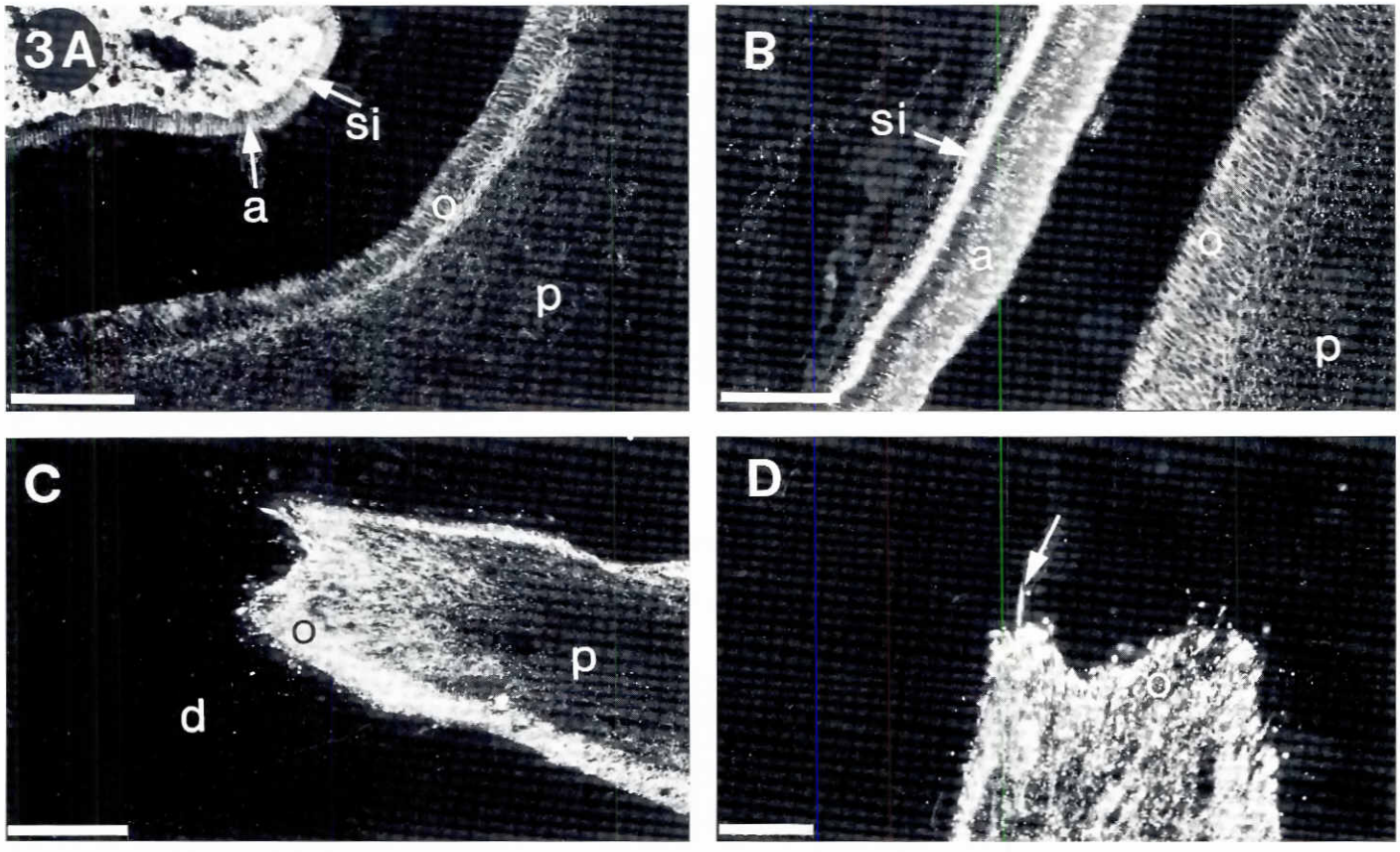


**Fig. 3. Connexin 43 immunoreactivity in the developing molar and incisor and the adult molar of the rat. (A)** *Detail of the cuspal area in a 5 day-old molar. A moderate immunoreactivity is present in the odontoblast layer (o) while the pulp (p) is devoid of staining. The ameloblasts (a) and the stratum intermedium (si) are immunolabeled, and the overlying stellate reticulum is strongly positive. Bar, 200 μm.* **(B)** *Detail of the posterior part of an incisor of a 5 day-old rat. Connexin 43 immunoreactivity is present in the stratum intermedium (si) and the ameloblasts (a), and somewhat weaker in the odontoblast layer (o). The pulp (p) is not labeled. Bar, 200 μm.* **(C)** *Pulpal horn of an adult rat molar. Connexin 43 immunoreactivity is strong in the odontoblast layer (o) but absent in pulpal mesenchyme (p). d, dentin. Bar, 200 μm.* **(D)** *Detail from a pulpal horn in an adult molar (adjacent section to* **C***). The odontoblasts (o) are strongly stained, and a labeled odontoblast process extends into the dentin (arrow). Bar, 100 μm.*

part of the pulp, staining was very faint or even absent. The same pattern of Cx43 expression in the odontoblastic region remained one week after a lesion of the inferior alveolar nerve (Fig. 4A). In tissues surrounding the teeth, Cx43-Ir was seen in the gingiva, where strong staining was found in the hypertrophic connective tissue underlying the junctional epithelium (Fig. 4B). In the periodontal ligament Cx43-Ir was moderate, whereas a strong staining was often found in the periapical tissue. Faint Cx43-Ir was detected in the loose connective tissue of the alveolar bone, and in the cell layer covering the internal surface of bone marrow crypts. (Fig. 4C). Cell clusters located on the root surface or close to bifurcations were occasionally positive for Cx43-Ir (Fig. 4D).

### *Expression of connexins in injured or aged rat teeth*

In teeth with a pulpal lesion (Fig. 4E,F,H), very strong Cx43-Ir was observed in the odontoblastic layer. This intense staining was also observed in the pulpal connective tissue invaded by macrophages. The staining was most prominent adjacent to the lesion, while Cx43-Ir was gradually decreased at sites away from the injury area. Pulpal injury did not change the expression of Cx32 or Cx26 (data not shown). In teeth from senescent rats aged 24 or 36 months, some teeth displayed an extensive calcification with total obliteration of the pulpal space (cf. Johansson *et al.*, 1996). However, in open pulps with an intact odontoblast layer, Cx43-Ir was similar to that seen in younger adult teeth (Fig. 4G).

### *Confocal and electron microscopy studies*

Double-labeling with Cx43- and CGRP-antiserum showed a dense arborization of CGRP-positive nerve fibers in the odontoblast region where Cx43-Ir was abundant. However, although occasional very close sites of contact were observed, there was no obvious co-localization of CGRP- and Cx43-Ir (Fig. 5).

Electron microscopy revealed that Cx43-Ir in the odontoblast layer was found primarily at sites of contact between odontoblasts, thus demonstrating locations of GJs (Fig. 6A-B). In addition there was a sparse labeling of odontoblast cytoplasm. Cx43-labeling was repeatedly observed at sites where fine cellular processes apposed odontoblast cell bodies. However, although such processes sometimes resembled nerve fibers (Fig. 6C), none of them could be identified with certainty as being of neural origin. Double labeling with Cx43 antibody and antiserum against PGP 9.5, a marker for nerve terminals in the dental pulp (Ramieri *et al.*, 1990), revealed that nerve fibers often were juxtaposed to Cx43-positive gap junctions, but clear-cut Cx43-immunoreactive axon-odontob-

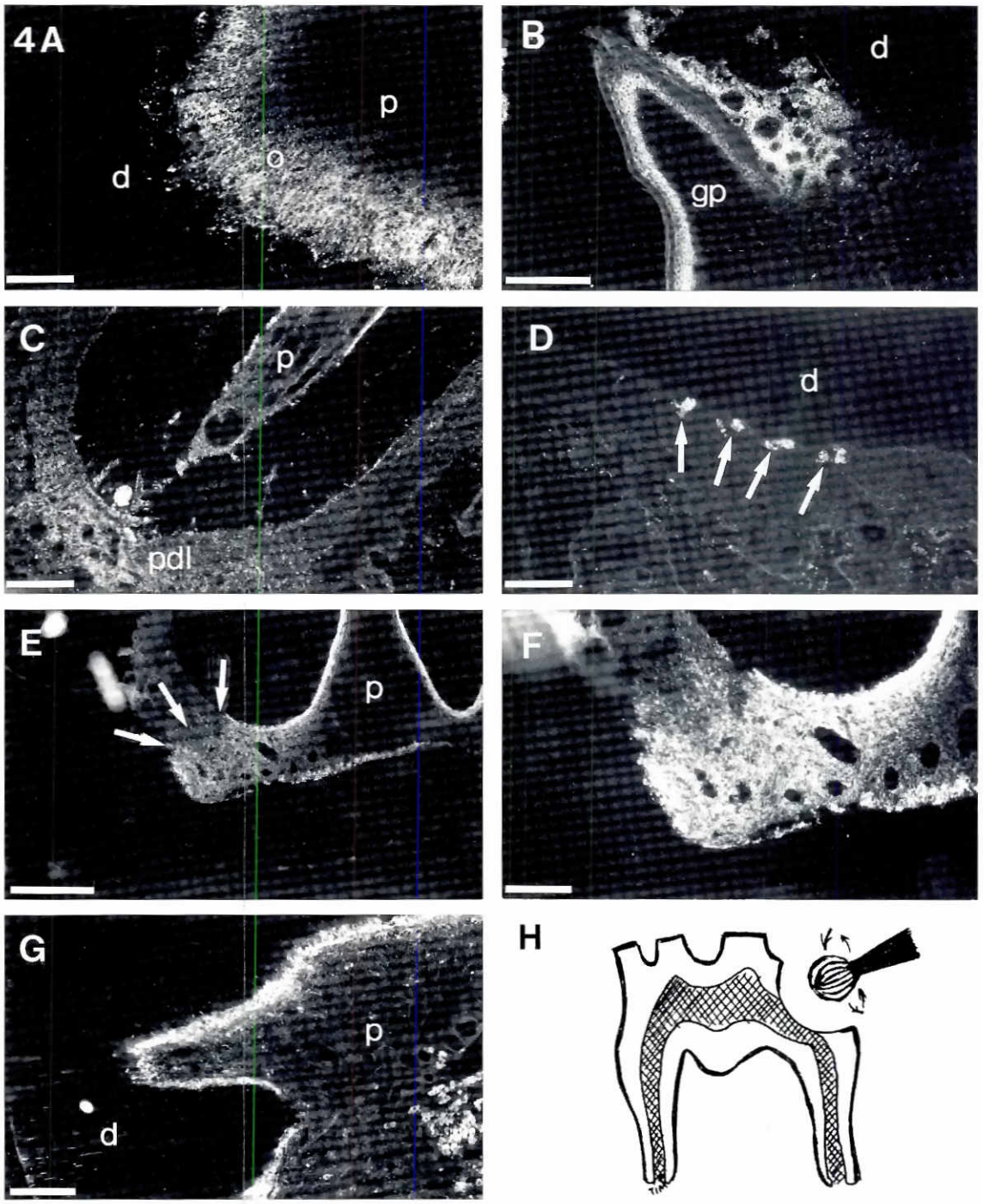


**Fig. 4. Connexin 43 immunoreactivity in the adult or aging molar of the rat. (A)** *The odontoblast layer (o) one week after denervation. Immunostaining is prominent and of a very similar distribution to that seen in the normal tooth. d, dentin; p, pulp. Bar, 100 μm.* **(B)** *Section through a gingival papilla (gp) and junctional region. Strong labeling is seen in the gingival epithelium and in hypertrophic connective tissue underlying the junctional epithelium. d, dentin. Bar, 200 μm.* **(C)** *The picture shows the periapical region of a molar root. Immunostaining is present in the odontoblast layer of the root pulp (p), in the periodontal ligament (pdl) surrounding the apex and in the cell layer covering the internal surface of bone marrow crypts. Bar, 200 μm.* **(D)** *Higher magnification of the periodontal ligament in the bifurcational region. Cell clusters (arrows) located on the root surface are connexin 43-positive for Cx43. d, dentin. Bar, 100 μm.* **(E)** *Molar pulp 4 days after exposure injury. Immunoreactivity is present in the odontoblast layer in intact regions of the pulp (p). In addition, the pulpal tissue beneath the exposure injury (arrows) is connexin 43-positive. This region is enlarged in* **(F)**. *Bar, 200 μm.* **(F)** *Higher magnification of the injured pulpal region shown in* **(E)**, *demonstrating a widespread immunostaining in the reactive pulpal mesenchyme. Bar, 100 μm.* **(G)** *Pulpal horn of an aging (24 months) rat molar. The odontoblast layer is stained in a pattern similar to that seen in the young adult tooth. d, dentin; p, pulp. Bar, 100 μm.* **(H)** *Schematic drawing showing the technique used to produce pulpal injuries. An occlusal-approximal surface cavity was made using a small rosehead bur in a slow running handpiece until pulp tissue was barely exposed.*

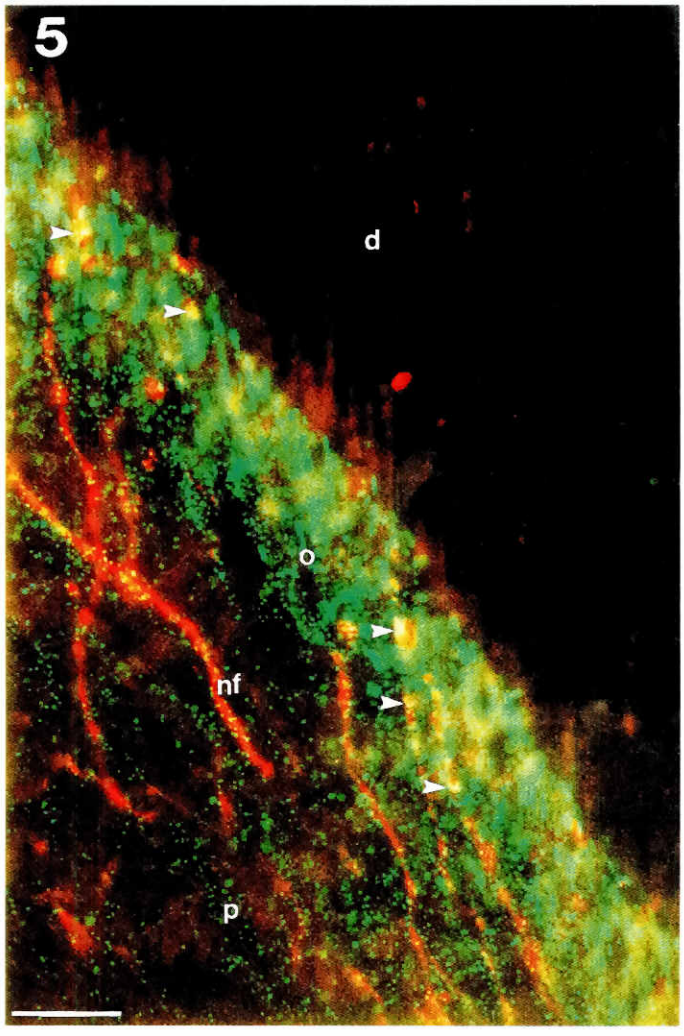


**Fig. 5. Confocal laser scanning image of a section of the pulp-dentin border in an adult molar after double incubation with connexin 43 antiserum and calciton gene-related peptide antiserum.** *The Connexin 43-positive odontoblast layer (o) is green, and nerve fibers (nf) positive for calcitonin gene-related peptide are red. Sites of very close contacts between Connexin 43- and calcitonin gene-related peptide can be observed (yellow, marked with arrowheads), although it cannot be determined with certainty that these are sites of co-localization of the two antigens. p, pulp; d, dentin. Bar, 100 μm.*

last or axon-axon contacts were not detected at the EM level (Fig. 7A-B).

### *Expression of connexins in cultured dental epithelial cells and pulpal fibroblasts*

Fibroblasts in cell culture derived from early postnatal dental papilla explants showed a moderate to strong Cx43-Ir. Extensive surface antigen fluorescence outlined the cells, often but not always, at cell-cell junctions. In addition, a dotted labeling was often present within the cytoplasm (Fig. 8A-B). There was no obvious change in Cx43-Ir in the cells when interleukin-1β was added to the medium. In the electron microscope, only little Cx43-Ir was present intracellularly, with the exception for occasional membrane-bound cytoplasmic bodies. Scattered Cx43-Ir was seen along cell membranes. At junctions between cell processes, however, a strong Cx43-Ir was evident in a pattern which was identical to that seen *in vivo* between odontoblasts (Fig. 8C-E).

Cultured dental epithelial cells from the cervical loop of the rat incisor (stem cells) exhibited both Cx32- and Cx43-Ir. The staining was mainly found in the surfaces of adjacent cells, but in some cases an intracellular labeling was observed (Fig. 8F-G).

## Discussion

The present data show a combinatorial expression pattern of connexins (Cx) in the developing and adult teeth of rodents. In dental epithelium, Cx43 is co-expressed with Cx32 during the polarization of preameloblasts/ameloblasts. In the mesenchyme, Cx43 is widely distributed in the odontoblastic layer from the earlier developmental stages and expression persisted during the lifetime of the animal. Cx32 is weakly expressed in differentiating odontoblasts, while Cx26 is absent from GJs formed between the odontoblasts. After pulpal injury there is a widespread Cx43 expression in fibroblastic cells. In cell cultures, pulpal fibroblasts express Cx43, while epithelial cells express both Cx32 and Cx43. Finally, GJs between axon/odontoblast and axon/axon are very rare, but do exist.

The GJs channels consist of different Cx (for review, see Bennett *et al.*, 1991), and each channel contains two hemichannels or connexons, each of which contains six identical protein subunits. A GJ may include two identical connexons (homotypic) or two connexons which differ with regard to the Cx (heterotypic). In excitable cells, GJs provide a means to exchange electrical signals. In cells that are inexcitable, GJs form a passage route for metabolites and regulatory molecules. To date, 12 different connexins have been cloned (Beyer *et al.*, 1987; Zhang and Nicholson 1989; Bennett *et al.*, 1991; Hoh *et al.*, 1991; Willecke *et al.*, 1991; Haefliger *et al.*, 1992; Hennemann *et al.*, 1992; Risek *et al.*, 1992; Kamibayashi *et al.*, 1993; Goliger and Paul, 1994). To some degree the connexins are specific for certain tissues. For instance, Cx36 and Cx32 are mostly found in epithelium-derived organs such as liver, pancreas and kidney (Paul, 1986; Traub *et al.*, 1989; Zhang and Nicholson, 1989), while Cx43 is expressed in heart, brain and mesenchymal tissue, e.g. endothelial cells and fibroblasts (Beyer *et al.*, 1987, 1989). The generation of specific antibodies against the connexins has provided a possibility to study GJ distribution at the light microscopical level, and given opportunities to examine the localization of Cx-identified GJs in the electron microscope. The presence of GJs in the odontoblast layer of the tooth pulp is known from previous EM transmission and freeze fracture studies (Köling and Rask-Andersen, 1981, 1984a,b; Calle *et al.*, 1985; Holland, 1987). The present study shows that Cx43 is an important constituent of odontoblast GJs. It is clear that the majority of these junctions connect adjacent odontoblasts, although the main function of such contacts has only been speculated upon. However, it has also been suggested that some GJs may form between terminal nerve fibers and odontoblasts, as well as between closely apposed nerve fibers (Holland, 1987). GJs between nerve fibers and odontoblast membranes would provide a substrate for interaction between these two structures, giving support to the hypothesis of the odontoblast as part of a specialized sensory receptor complex. In addition, the existence of possible GJs between adjacent axons has been inferred on the basis of electrophysiological data (Matthews and Holland, 1975).

Cx43 expression was widespread in the stratum intermedium, inner enamel epithelium and odontoblast layer from the earliest developmental stages examined here, in accordance with recently reported findings (Kagayama *et al.*, 1995). It has been suggested that during development GJs could provide a pathway for passage of various molecules and ions between adjacent cells, at a stage when vascularization is insufficient for nutrient supply (Caveney, 1985; Risek *et al.*, 1994). This could be the main function of GJs between odontoblasts, cells which have a period of intense synthesis and secretion during dentinogenesis. Furthermore, the odontoblasts act as a semi-permeable barrier between the pulpal extracellular space and the predentin, to allow for a proper milieu for calcification in the predentin (Turner *et al.*, 1989). GJs may, e.g. by allowing spatially graded distributions of molecules (Caveney, 1985), be important in maintaining odontoblast position and polarity so that this barrier remains intact during dentin formation and pulpal maturation. In old animals, we found no obvious signs of a reduction in odontoblast GJ density. Aging frequently involves both secondary dentin formation and regressive changes of the odontoblast layer (Couve, 1986; Fried, 1992). However, the present findings indicate that when the odontoblasts remain intact in senescence, they also retain their ability to maintain GJ contacts with neighboring cells.

Cx43 expression *in vivo* was prominent in pulpal connective tissue under a pulpal lesion, in contrast to normal pulpal mesenchyme which was devoid of Cx43. The physiological significance and mechanism for this increase in synthesis is not clear. Formation of GJs between the pulpal fibroblasts may enable an exchange of metabolites which otherwise become less available due to e.g. disturbances of the local blood flow. Furthermore, the GJs may play a role in the defense reaction by linking fibroblasts, thus creating a diffusion barrier which protects the deeper pulpal regions. GJ transcripts are modulated by steroid hormones (Risek *et al.*, 1990), but it appears unlikely that hormones are involved in pulpal injury-related GJ upregulation. It is possible that macrophage-related substances, e.g. cytokines, could be responsible for the increase in Cx43 synthesis after pulpal injury. Addition of the cytokine interleukin-1β to pulpal fibroblasts *in vitro,* though, did not induce any noticeable change in Cx43 expression. However, since the pulpal fibroblasts *in vitro* generally expressed Cx43, unlike those *in vivo*, a possible interleukin-1β-effect would be difficult to discern in the system we have used here.

Several previous studies have pointed out the close connection between odontoblasts and nerve fibers (see Hildebrand *et al.*, 1995 for references). The possibility exists that some of the GJs in this region link nerve fibers with odontoblasts or with adjacent nerve fibers. However, this issue has been difficult to settle with structural methods because of the problem to identify terminal, sometimes unensheathed, axons in this zone. Such junctions, which would represent linkages of low electrical resistance, could have important roles in sensory transduction from the pulp. Thus, they could be of significance for odontoblast-neural modulation and/or electrical coupling between nerve fibers. However, although occasional co-localization of Cx43 and CGRP immunoreactivity was demonstrated in this study, our denervation experiments and EM immunocytochemistry do not provide further evidence for such contacts. In fact, it cannot be excluded that the occasional Cx43 and CGRP-co-localization may be represented by overlaying immunoreactivities, i.e. not true co-localization contacts between nerve fibers and odontoblasts. This does not exclude the possibility that terminal

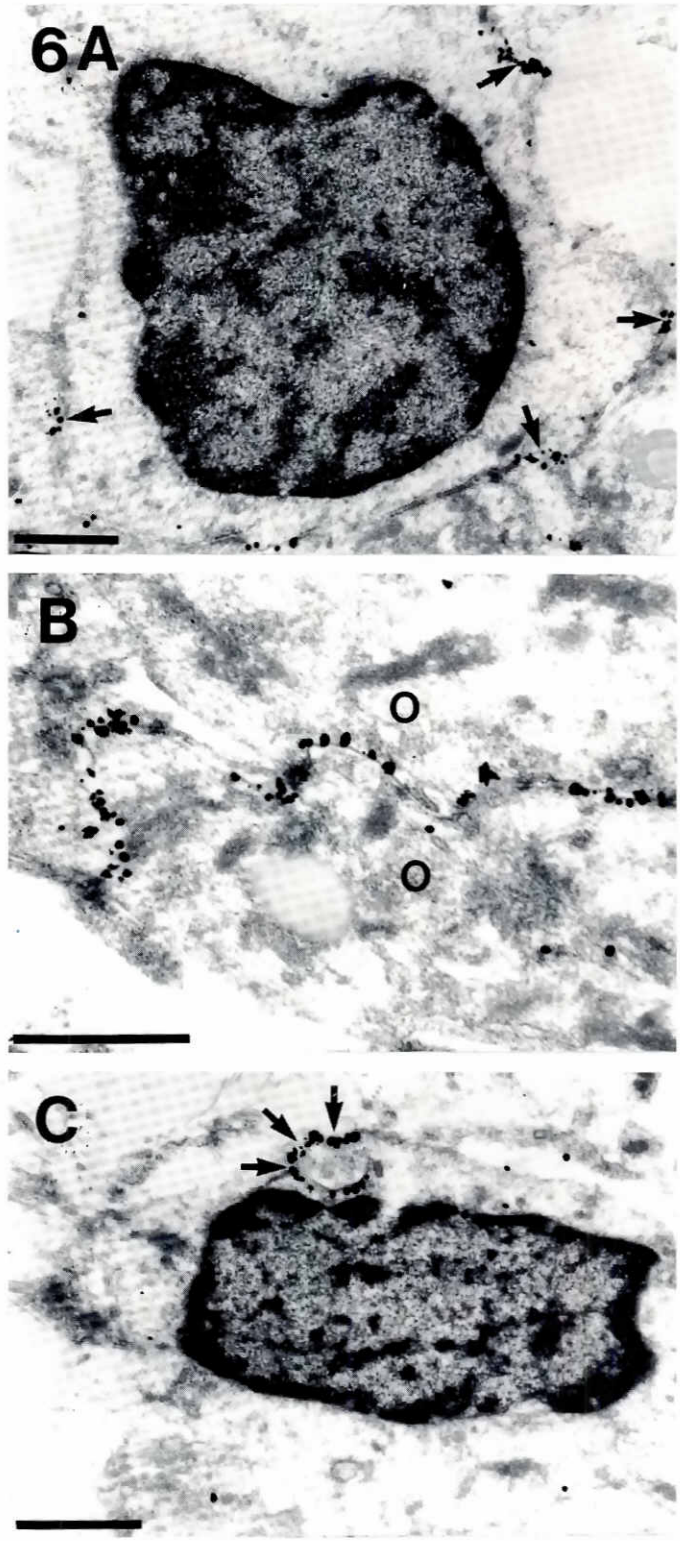


**Fig. 6. Electron micrographs showing connexin 43 immunogold-silver labeled odontoblasts in adult rat molars. (A)** *Odontoblast with several immunostained contacts (arrows) with surrounding odontoblasts.* **(B)** *Higher magnification of an immunolabeled gap junction contact between two odontoblasts (o).* **(C)** *An example of an immunostained contact between an odontoblast and a cellular process reminiscent of a nerve fiber (arrow). Bars, 1 μm.*

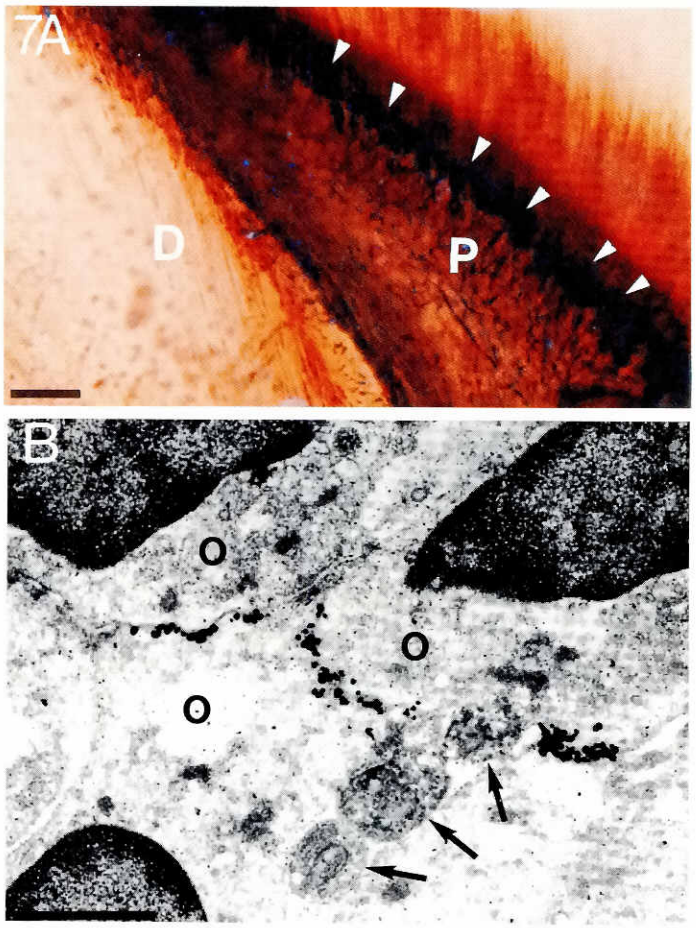


**Fig. 7. Micrographs showing an adult molar after double incubation with connexin 43 antiserum and PGP 9.5 antiserum. (A)** *Light micrograph of a 50 μm plastic embedded section simultaneously viewed in transmitted light and incident polarized light. The picture shows a dense pulpal network of PGP 9.5-immunoreactive nerve fiber arborizations labeled by HRP reaction product (dark brown). The nerve fibers branch into the odontoblast layer, which is Cx43-immunogold-positive. The immunogold-silver particles are dark brown in transmitted light, but in addition they emit a bluish fluorescence (arrows) in the polarized light. P, pulp; D, dentin.* **(B)** *Electron micrograph of an ultrathin section from the specimen in* **A**. *The micrograph displays PGP 9.5-immunoreactive nerve fibers labeled with HRP reaction product (arrows) in close proximity to Cx43 immunogold-positive gap junctions. No HRP reaction product is visible in odontoblasts (o). Bars: A, 100 μm; B, 1 μm.*

axon-axon contacts could be formed by GJ proteins other than Cx43, Cx26 or Cx32. Such junctions may hypothetically link nerve fibers with odontoblasts as well, provided that odontoblasts synthesize Cx additional to those studied here. Heterotypic junctions that join Cx43 hemichannels on odontoblasts with a different Cx type of hemichannel on an axon (see Kumar and Gilula, 1992) probably do not exist, since they most likely would have been recognized as Cx43-positive structures in the present material. It may be worth mentioning that an ephaptic crosstalk between adjacent nerve fibers could occur even though GJs between them are absent, provided that their membranes appose for long enough distances (Fried *et al.*, 1993). Hence, our structural findings do not exclude the possibility of electrical coupling between pulpal axons. Finally, although GJs rarely attach odontoblasts to nerve fibers, the upregulation of odontoblast and/or connective tissue Cx43 after pulpal exposure could indirectly affect pulpal nerve fiber function by changing the extracellular milieu of preterminal-terminal pulpal axons. In this way, alterations in pulpal GJ density could perhaps be a factor in the etiology of injury-induced pulpal or dentinal hypersensitivity (Närhi *et al.*, 1994).

## Materials and Methods

### *Animals and tissue preparation*

For studies on the developing teeth, Wistar rat embryos (E20-21) and newborn rats (PN1-12) were sacrificed. The animals were anesthetized with ether and then perfused as described above. The mandibles from the E20-21 embryos and the PN1-3 day-old animals were sectioned without prior decalcification. The mandibles from the PN5-12 day-old animals were decalcified for one week as described above.

For studies on the normal, denervated and injured adult teeth, adult and old (24 and 36 months) male Wistar rats (b.wt. 250-300 g and 550-600 g, respectively), were used. Three of the adult animals underwent inferior alveolar nerve (IAN) section. The rats were allowed to recover and survive for one week. In three additional rats, a pulpal lesion of the first left mandibular and maxillary molar was made with a small bur, and subsequently the animals were allowed to survive for one week. All animals were then reanesthetized and perfused with Tyrode's solution (37°C) followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. After perfusion, both mandible halves were dissected out, post fixed for 90 min in the fixative and then transferred to 10% sucrose buffer solution overnight. The molar teeth were removed in a block of alveolar bone and decalcified in 4% ethylenediamine tetra-acetate (EDTA, Titriplex®, Merck, Germany) in cacodylate buffer for 6 weeks.

All teeth were cut in a bucco-lingual plane into 14 μm thick sections on a cryostat (Microm, Germany). The sections were mounted on glass slides coated with chrome alum gelatin and then processed for indirect immunofluorescence histochemistry.

### *Immunohistochemistry*

The sections were rehydrated and incubated with a site-specific monoclonal mouse antibody to connexin (Cx) 43 (Affiniti, Nottingham, UK, dilution 1:500) or with polyclonal affinity-purified antibodies against Cx43, Cx32, and Cx26 (see Meda *et al.*, 1993; Guerrier *et al.*, 1995; dilution 1:100, antibodies generously supplied by Dr. D. Gros, Marseille, France) for 24 h in a humid atmosphere. The production and characterization of these antibodies has been reported previously (Guerrier *et al.*, 1995). Some sections were incubated with a mixture of the monoclonal Cx43 antibody and rabbit polyclonal antibodies to CGRP (see Fried *et al.*, 1989; dilution 1:100, antiserum generously supplied by Dr. E. Theodorsson). After incubation with the primary antiserum, the sections were rinsed in 0.1 M phosphate-buffered saline (PBS) and then incubated with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (dilution 1:10, Dakopatts, Denmark), or with rhodamine-conjugated sheep-anti-mouse antibodies (dilution 1:100, Boehringer Mannheim, Indianapolis, IN, USA), which in double labeled specimens were mixed with FITC-conjugated goat anti-rabbit antibodies, for 45 min. Control sections were incubated either using a preabsorption test in which the primary Cx43 antibody was mixed with the corresponding Cx43 peptide (10-fold excess by weight; Affiniti, Nottingham, UK), or by omitting a primary antibody. Both these procedures abolished immunostaining. All sections were mounted in a mixture of PBS and glycerol (1:3), coverslipped and examined by use of either a Leitz DM RBE microscope equipped with the appropriate filter combinations to view FITC or rhodamine fluorescence. For photography, Kodak T-Max 400 was used. In addition, some double-labeled sections were examined in a BIO-RAD MRC-600 laser scanning confocal imaging system equipped with a Krypton/Argon mixed gas laser and a Nikon Optiphot II microscope. The standard BHS single channel filter block with an excitation filter (488 DF 10 for FITC-induced fluorescence and a 568 DF 10 for rhodamine-induced

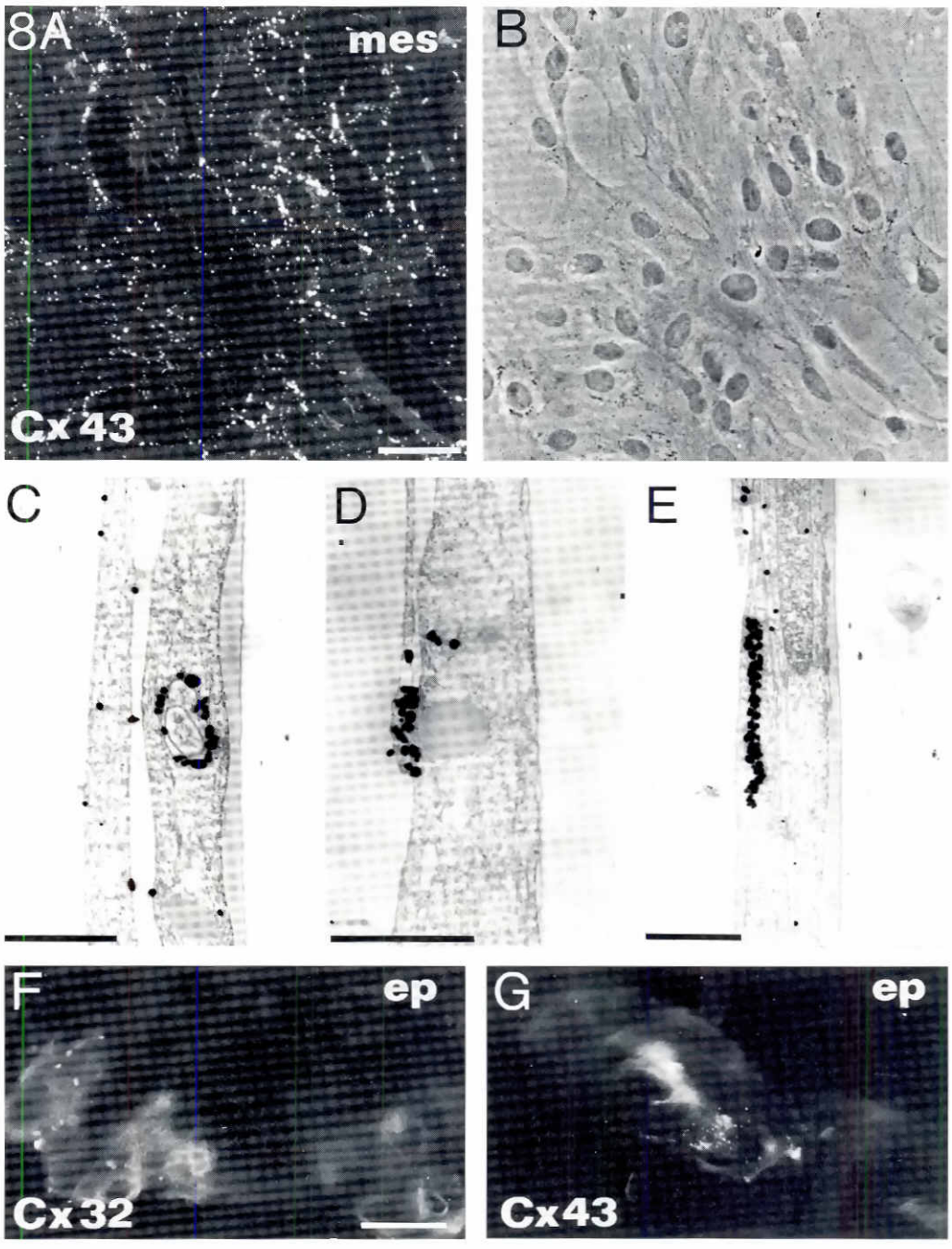


**Fig. 8. Light- and electron micrographs of connexin-immunoreactive cultured pulpal fibroblasts and dental epithelial cells. (A)** *Pulpal mesenchymal fibroblasts (mes) in vitro, showing intense Connexin 43-immunoreactivity, primarily at sites of contacts with other cells. Bar, 50 μm.* **(B)** *Phase-contrast micrograph of the region depicted in* **(A)**. **(C-E)** *Electron micrographs of immunogold-silver labeled connexin 43 positive contacts between cultured pulpal fibroblasts. Note that these are very similar to the types of contacts observed in vivo (see Fig. 6). Bars in C-E, 1 μm.* **(F)** *Connexin 32 is distributed in contacts of dental epithelial cells (ep) of the rat incisor, but also in their cytoplasm. Scale bar (also applies to G): 20 μm.* **(G)** *Connexin 43 immunoreactivity is evident in dental epithelial cells (ep).*

fluorescence) was used to examine the immunoreactivity. The images were printed using a Tektronix Phaser II sd printer.

***Electron microscopic immunohistochemistry***

For pre-embedding EM immunocytochemistry molar teeth with surrounding bone were cut into 50 μm-thick cryostat sections, rinsed in PBS and immersed in Cx43 antiserum as described above. Some sections were incubated with a mixture of the monoclonal Cx43 antibody and rabbit polyclonal antibodies to the nerve fiber marker PGP 9.5 (UltraClone, Isle of Wight, UK). The sections were rinsed and double labeled specimens were incubated with a mixture of 1 nm gold particle-conjugated goat anti-mouse antibodies (Aurion) and biotinylated goat-anti-rabbit antibodies (Vector Labs., USA) and rinsed again. The double-labeled sections were then incubated with Vectastain ABC reagent (Vector Labs., USA), rinsed and subsequently incubated in peroxidase substrate solution (DAB, Sigma) and rinsed again. Single-labeled sections were incubated with gold particle-conjugated goat anti-mouse antibodies only, and all specimens were then osmicated by a modification of the Marchi method. Finally, the sections were intensified by use of a silver enhancement reaction (Intense M, Janssen Biotech.) and embedded in Vestopal W between transparent acetate foils. This permitted examinations at the light microscopical level, and selected regions were then sectioned by use of an LKB Ultrotome, contrasted with uranyl acetate and lead citrate and examined in a Philips CM12 electron microscope.

***Cultures of dental epithelial and mesenchymal cells of the rat***

For cell culture, dental epithelial cells from the cervical root of the rat incisor were harvested and cultivated according to the method described by Farges *et al.* (1991). For studies of dental pulp cells, rat pups (age 4-6 days postnatally) were decapitated and first molar teeth were dissected out from both jaws under sterile conditions. Pulpal tissue was dissociated from the mineralized caps and each pulp was cut into 3-5 pieces. The explants were cultured (1 ml/dish) in a medium containing DMEM (Dulbecco's Modified Eagle Medium): F12 Ham's Medium (4:1, Gibco), Epidermal Growth Factor (EGF) (10 ng/ml, Sigma), insulin (5 μg/ml, Sigma), transferrin (5 μg/ml, Sigma), triiodothyronine ($2x10^{-9}$ M, Calbiochem), hydrocortisone (2 μg/ml, Sigma), cholera toxin ($10^{-10}$ M, Sigma) and 10% Fetal Calf Serum (FCS) (Gibco), and incubated for 5-7 days before passaging. The primary outgrowth of cells consisted of fibroblast-like and epithelial-like cells, which were separated by short-term trypsinization (see Bongenhielm *et al.*, 1995).

***Immunohistochemistry on cultured dental cells***

For immunohistochemistry, fibroblast-like cells of 3rd to 6th passage cells were detached and replated onto 4 well Permanox slide plates (Nunc, IL, USA) and allowed to attach overnight in culture medium. In some cases, interleukin-1β (100 U/μl; R&D Systems, Abingdon, UK) was added to the medium, and all cells were then incubated for 12 h. Subsequently, the cells were rinsed first in fresh medium, then in PBS and then fixed for 30 min with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. After a rinse in the buffer, the cells were incubated with Cx43 antiserum as above, rinsed in 0.1 M phosphate buffer, incubated with Cy3-conjugated donkey-anti-mouse antibodies (dilution 1:100, Jackson ImmunoChemicals, USA), rinsed again and coverslipped. Controls and microscopic examinations were performed as described above. For electron microscopy, cells were replated onto Falcon Cyclopore membranes (pore size 45 μm; Becton Dickinson, NJ, USA) and incubated for 36 h. The cells were then rinsed, fixed and incubated with primary antiserum as above for fluorescence microscopy. Incubation with secondary gold-conjugated antibodies, silver intensification and further processing and sectioning of the cell-covered membrane for EM followed the protocol described above.

For immunohistochemistry on dental epithelial cells both Cx32 and Cx43 antibodies were used.

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