Neuro-Odontoblast Relationships

Tom Struys[1], Tracy De Peralta[2], Frank Vandenabeele[1], Wolfgang Hans-Michael Raab[3], Constantinus Politis[4], Serge Schepers[4], Luc Vrielinck[4], Ivo Lambrichts[1]

Objective: *To study the close relationship between odontoblasts and intradental nerves in order to elucidate its role in the sensory mechanism that is responsible for dentinal pain.*
Methods: *Jaws dissected from 40 mice and 35 rats, and 24 human molar teeth, were routinely embedded into paraffin or araldite®. Following embedding, the specimens were studied using immunohistochemistry for some common neuronal and neuropeptide markers or electron microscopy.*
Results: *Strong immunoreactivity for synaptophysin reveals the presence of presynaptic vesicles in the odontoblast zone of the pulp. Immunoreactivity for nerve growth factor receptor (NGFR) shows clearly that the neurites enter the dentinal tubules together with the odontoblast processes as they share a similar course. Electron microscopy images of the neuro–odontoblast relationship demonstrate some morphological characteristics such as synapse-like contacts between neurites and odontoblast processes, the presence of numerous small granular vesicles (SGV) and some clear cored vesicles in the odontoblast process at the synaptic contact and a typical synaptic cleft, 15 to 20 nm in width. From all these features, we can describe these neuro–odontoblast relationships as synaptic, based on immunological and morphological characteristics.*
Conclusion: *Although it has previously been proposed that odontoblasts also could act as a specialised receptor cell, to date no synaptic or gap junctions connecting them with the nerve fibres have been described. In this investigation of mouse, rat and human odontoblasts and intrapulpal and intradentinal nerves, we demonstrated the presence of chemical synapses using electron microscopy and immunohistochemistry.*
Key words: *immunohistochemistry, neural endings, synapse, odontoblast, transmission electron microscopy*

Dentine, the hard tissue portion of the pulp–dentine complex, forms the bulk of the tooth. Structurally it consists of multiple closely packed dentinal tubules, which traverse the entire thickness of the tooth. These tubules contain cytoplasmatic extensions of the odontoblasts as well as some fine neurites, which extend only into the pulpal part of the dentine[1]. The cell bodies of the odontoblasts form the boundary between the inner aspect of the dentine and the periphery of the dental pulp. The function of odontoblasts is not limited to dentinogenesis, but includes maintenance of dentine, and their important role in reparative dentine formation[2].

The interaction between the odontoblasts and the neurites, which are wrapped around the cell bodies of the odontoblasts and enter the dentinal tubules, might be responsible for dentine sensitivity. Numerous papers have been published on morphological, physiological and pharmacological characteristics of the nerve fibres in the dental pulp. Some of these suggested a possible recep-

1 Biomedical Research Institute, Hasselt University, Campus Diepenbeek, Belgium.
2 Peninsula Dental School, University of Plymouth, United Kingdom.
3 Department of Operative/Preventive Dentistry and Endodontics, Heinrich Heine University of Düsseldorf, Germany.
4 Maxillofacial Surgery, ZOL, Genk, Belgium.
Corresponding author: Dr. Tom STRUYS, Hasselt University, Campus Diepenbeek, Lab of Histology. Agoralaan, Building D, Office G0.54, 3590 Diepenbeek, Belgium. Tel: +3211 26 85 27; Fax: +3211 26 85 99. E-mail: tom.struys@uhasselt.be.

tor role for the odontoblast, but to date no synaptic or gap junctions, connecting them with the nerve fibres, have been described[3-7]. Advances in immunohistochemistry have revealed the existence of various neuropeptides in sensory and autonomic nerves in the dental pulp[8-11]. Understanding of the functional roles of the neuropeptides is still incomplete. Besides their functional role in peripheral pain perception and mechano-transduction (e.g. calcitonin gene-related peptide [CGRP], substance P, neurokinin A), their vasoactive properties (e.g. norepinephrine [NE], acetylcholine [ACh], neuropeptide Y [NPY], substance P) are very important. Some neuropeptides have other roles, such as in endocrine and exocrine secretion, bone remodelling, nerve regeneration, influence on immune response and their very important task in wound healing and dentine repair. Interactions between odontoblasts and the neurons innervating them involve more than the mechanisms of sensory and motor transduction. There are mutual trophic dependencies regulating structure and function of both partners (e.g. hepatocyte growth factor [HGF], nerve growth factor [NGF], brain-derived neurotrophic growth factor [BDNF], glial cell line-derived neurotrophic factor [GDNF])[12-13]. In a recent study, Magloire et al[14] demonstrated the presence of primary cilia on odontoblasts and suggested a possible receptor function for this organelle. These findings bring renewed interest in a possible receptor function of odontoblasts. The present investigation focuses on the morphological and immunohistochemical characteristics of neuro-odontoblast interactions.

Materials and Methods

Animals and materials

Forty mice, thirty-five Wistar rats and twenty-four human molar teeth were used in this study. The human teeth were permanent molars that were extracted for orthodontic or therapeutic reasons with informed consent of the patients.

Paraffin sections

HGF (Santa Cruz Product No: sc-7949, California, USA), nerve growth factor receptor (p75) (NGFR; Dako Product No: M3507, Carpintera, CA93013, USA), Synaptophysin (Dako Product No: M0776, Carpintera, CA93013, USA), NPY (Affiniti Product No: NA1233, Mamhead Exeter EX6 8HD, UK) and neurofilament (Dako Product No: N1591, Carpintera, CA93013, USA) immunostainings were performed on paraffin sections. The pulp of the human teeth was carefully removed, preserved overnight in a 4% formaldehyde solution and then put through dehydrating series of graded concentrations of alcohol and finally embedded in paraffin. Paraffin sections (5–8 μm) were attached to poly-L-lysine coated glass slides. Sections were deparaffinised with xylol, followed by decreasing concentrations of alcohol and then washed in PBS. Endogenous peroxidase activity was quenched with 0.5% H_2O_2. Non-specific binding sites were blocked with 3% normal goat serum. Subsequently, sections were incubated with mouse monoclonal antibodies against the proteins mentioned above. Sections were incubated with a peroxidase-labelled polymer conjugated to a goat anti-mouse secondary antibody for 30 min (Envision System®, DakoCytomation, Glostrup, Denmark). Immunoreactivity was visualised with diaminobenzidine (DAB). Sections were counterstained with hematoxylin, coverslipped with an aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany) and examined using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).

Frozen sections

The study was carried out with 35 Wistar rats in which jaws were dissected and sliced into approximately three 1 mm-thick sections using a diamond-cutting horizontal saw (Leica SP 1600, Nussloch, Germany) and a sterile water coolant at room temperature. Slices were placed in 4% formaldehyde for 24 hours, washed in phosphate buffer for 24 hours and then placed in 25% EDTA at 4°C on a slow rotating mixer (IKAR Vibrax VXR basic, Janke & Kunkel, Staufen, Germany) for 7 days to decalcify.

Immunohistological investigation was carried out using antibodies against CGRP (Sigma-Aldrich Product No. C8198, Saint Louis, Missouri, USA), periferin intermediate filament protein (PER; UltraClone Limited Product No. 31[A], Isle of Wight, England), and protein gene product 9.5 (PGP 9.5; Chemicon Product No. AB1530, Munich, Germany). Immunohistochemical labelling was carried out on 30 μm cryosections of jaw slices using a Vectastain pK4001-kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions with some modifications. Briefly, sections were washed in 0.01 mol/l PBS and incubated with 0.3% H_2O_2 in PBS for 30 min at 4°C. After PBS washes, the sections were incubated with normal goat serum in PBS for 1 h at room temperature. Subsequently, serial sections were divided and incubated in polyclonal rabbit anti-CGRP serum (1:10,000), polyclonal rabbit anti-PGP 9.5 (1:15,000), or polyclonal rabbit an-

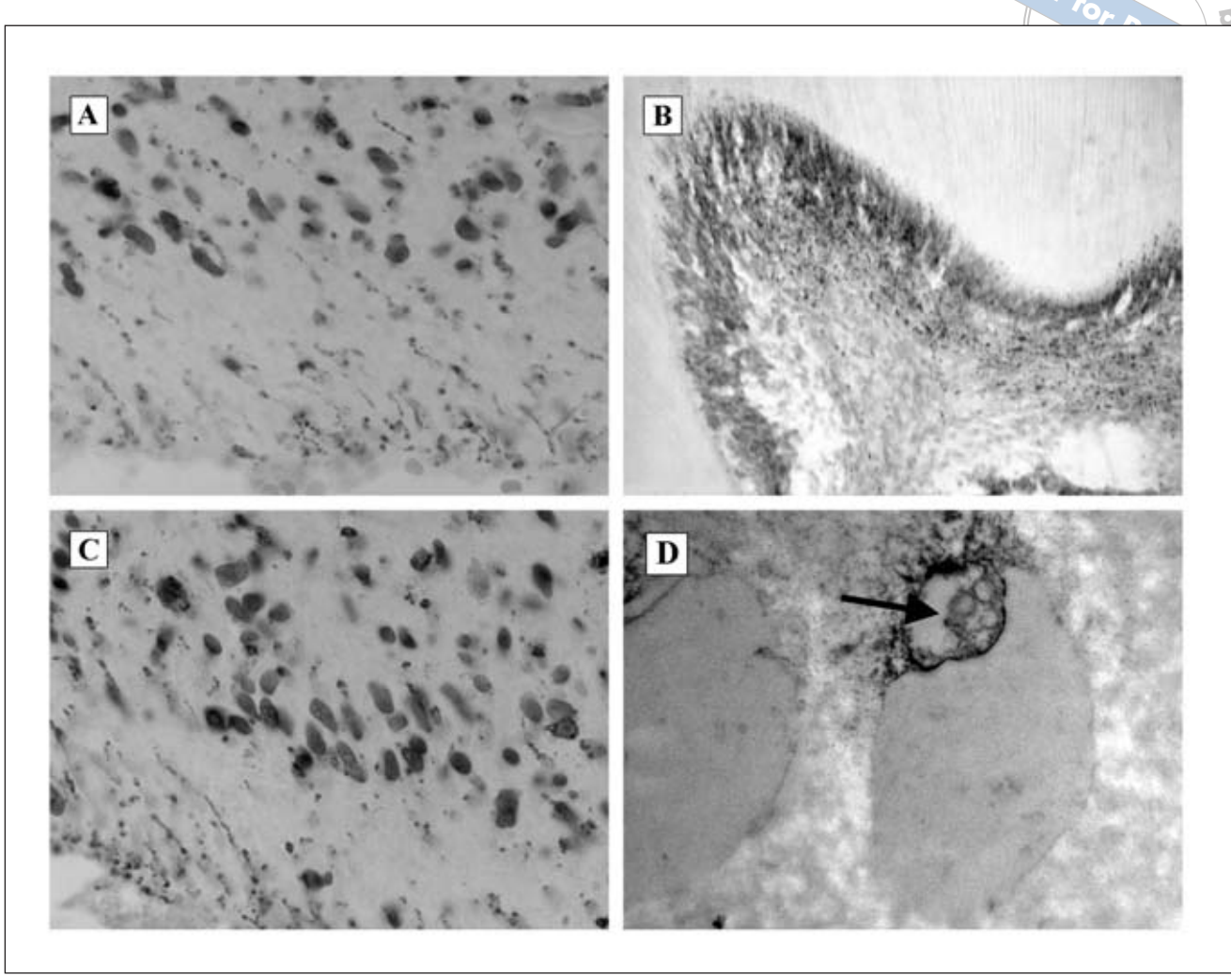

Fig 1 Immunohistochemistry and immuno-EM for synaptophysin (A), calcitonin gene-related peptide (B) and nerve growth factor (C and D). These antibodies specifically mark nerve fibres and they show the simultaneous course of these fibres with the odontoblast processes. Note in (D) that the membrane of the odontoblast itself is also positive for nerve growth factor and this is only at the contact site with the nerve fibre. Vesicles are present in the nerve fibre (arrow).

ti-PER (1:8,000) (all diluted in PBS containing 1% BSA [Sigma, Steinheim, Germany] for 72 h at 4°C). A negative control of the immunohistochemical reaction was carried out by incubating sections where the primary antibody was omitted. After PBS washes, biotinylated goat secondary antibodies against rabbit IgG (Vector Laboratories, Burlingame, CA, USA) were applied onto the sections for 1.5 h at room temperature. After further PBS washes the sections were incubated for 4 h with an avidin-biotin-peroxidase complex. Final visualisation was made with 0.2% nickel-enhanced 0.05% diaminobenzidine (Sigma, Saint Louis, USA) and 0.003% H_2O_2. Sections were then mounted onto gelatin-coated slides and counterstained.

Transmission electron microscopy (TEM)

For examination by TEM, human pulp and NGFR, neurofilament, S100 (Dako Product No: Z0311, Carpintera, CA93013, USA) and glial fibrillary acidic protein (GFAP; Novocastra Product No: NCL-GFAP-GA5, Newcastle upon Tyne, UK)-immunostained rat and mouse jaw slices were fixed overnight in a solution containing 2% glutaraldehyde in 0.05 mol/l cacodylate buffer (pH 7.3) at 4°C, postfixed in 2% osmium tetroxide for 1 h, stained with 2% uranyl acetate in 10% acetone for 20 min, put through a dehydrating series of graded concentrations of acetone and embedded in epoxy resin (Araldite®). Semi-thin sections were prepared to

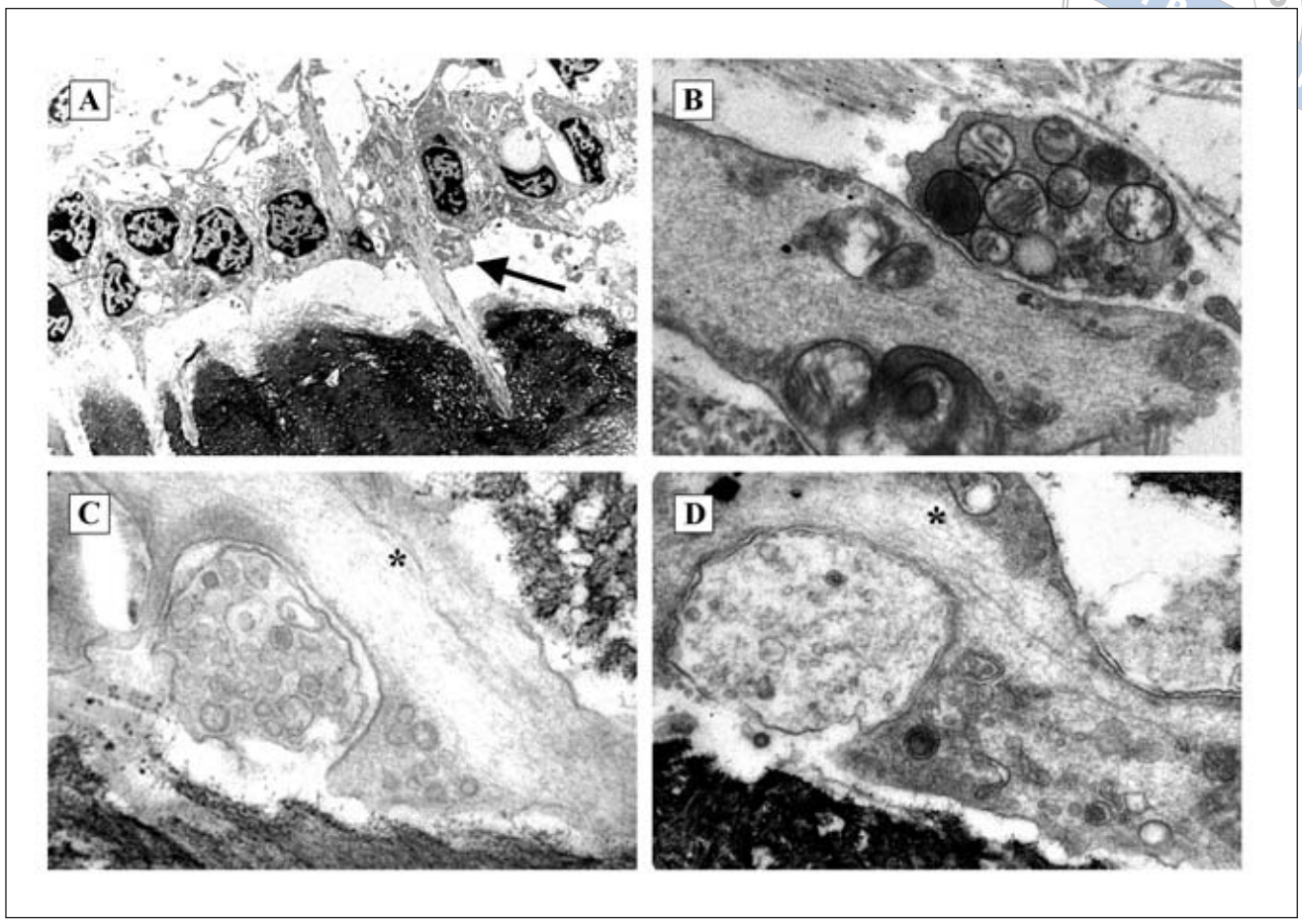

Fig 2 EM images of the neuro–odontoblast relationships. Image (A) and (B) show close apposition of nerve fibres and odontoblast processes (arrow in A). Images (C) and (D) show the synapses between nerve fibres and odontoblast processes (the latter are indicated *). These structures contain all the elements of a classic morphological synapse.

select areas of interest. Serial ultra-thin sections (0.06 μm) were mounted on 0.7% formvar coated grids, contrasted with uranyl acetate followed by lead citrate, and examined in a Philips EM 208 transmission electron microscope operated at 80 kV.

Results

Immunohistochemistry

All specimens showed immunoreactivity for all the antibodies used in this study. Immunostaining for synaptophysin, CGRP and NGFR are shown in Fig 1. Strong immunoreactivity for synaptophysin reveals the presence of presynaptic vesicles in the odontoblast zone of the pulp (Fig 1A). CGRP and NGFR immunoreactivity shows the extensive branching of nerve fibres that is typical for the odontoblast zone (Figs 1B and 1C). The NGFR immunoreactivity shows clearly that the neurites enter the dentinal tubules together with the odontoblast processes as they share a similar course (Fig 1A). Electron microscopic immunohistochemistry for NGFR shows that the neurites in the dentinal tubules express this receptor. Also the membrane of the odontoblast is positive for NGFR, and this only at the contact zone with the neurite (Fig 1D). Other immunostainings are not shown, but contributed to the detection of neuro–odontoblast interactions.

Immunoreactivity for HGF was seen in the odontoblast layer of the dental pulps. The cell bodies of the odontoblasts exhibited the strongest staining. The odontoblast processes were stained to a lesser degree.

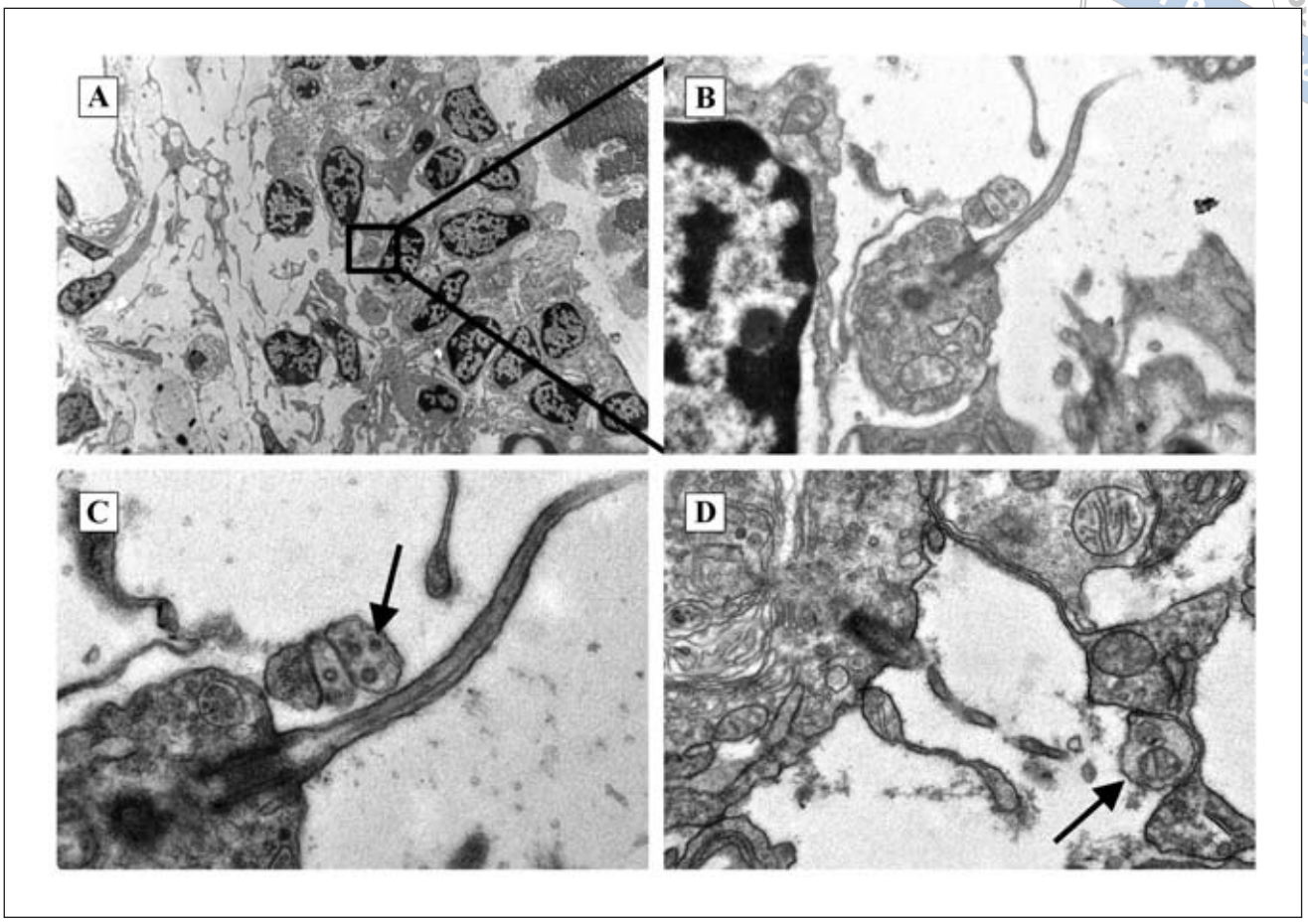

Fig 3 EM images of cilia present on the odontoblast cell bodies. Images (A) and (B) show the orientation of the cilia which was mainly towards the pulp centre (blood vessels and neural tissue). Images (C) and (D) show the close relation between these cilia and neural tissue (arrows) which is devoid of Schwann cell cytoplasm.

Electron microscopy

The cytological characteristics and functions of human odontoblasts are generally very similar to those of rodents. The nucleus is near to the base of these tall cells (Fig 2A), and there is a large Golgi-apparatus between the nucleus and the cell apex. The supranuclear region also contains many cisternal profiles of rough endoplasmatic reticulum oriented more or less parallel to the long axis of the cell. Free ribosomes are also abundant. A tapering odontoblast process extends through the non-mineralised predentine and into tubular channels in the mineralised dentine matrix.

The cytoplasm of the odontoblastic process is mostly devoid of membranous organelles. The cells actively synthesise and secrete precursors of collagen and the amorphous glycosaminoglycans. Neural profiles were frequently encountered in close apposition to the odontoblast processes and cell bodies. In general, the neural profiles have an electron lucent cytoplasm while the odontoblast processes are more electron dense (Fig 2A). Mitochondria are present in neural profiles and in the odontoblast processes also at the level of the contact area (Fig 2B). Neural structures are observed at the transition-zone between the odontoblast process and cell body (Fig 2A). In the subodontoblastic layer, the neurites are accompanied by Schwann cell processes. In the predentine and in the dentine these neural profiles are devoid of the Schwann cell sheath. It is obvious that the odontoblasts take over the role of the Schwann cells in supporting the neurites. Figs 2C and 2D demonstrate the synapse-like contacts between the light-staining neurite and the dark-

staining, filament rich, odontoblast process. The latter contains numerous small granular resicles (SGV) and some clear cored vesicles at the synaptic contact. The neural profiles also contain SGV, large granular vesicles (LGV) and clear cored vesicles. The synaptic cleft measured 15 to 20 nm in width. From all these features, we can describe these neuro–odontoblast relationships as synaptic, based on morphological characteristics.

In this study, primary cilia were found on several odontoblasts. In contrast to the primary cilia described by Magloire et al[14], the orientation of most of the cilia was projected towards blood vessels and nervous tissue in the pulp (Figs 3A and 3B). The basal bodies of the cilia were located in the infra-nuclear region of the cytoplasm of the odontoblast. In five specimens we observed a close apposition of cilia and neural varicosities devoid of Schwann cell covering (Figs 3C and 3D).

Discussion

The odontoblast process is not the sole occupant of the dentinal tubules. In some areas many of the processes are accompanied for part of their course by sensory axons[15-16]. The most favoured explanation for the so-called hydrodynamic mechanism does not require the presence of nerves or receptors in the dentine.

Frank et al[16] showed a complex relationship between a nerve-like structure and an odontoblast process and described it as an intimate functional relationship between a connective tissue cell process and a sensory nerve fibre: a unique neurosensitive complex. Arwill et al[4] described similar structures and remarked on their synapse-like appearance. Enthusiasm for this special relationship has not thrived. Byers et al[17] found 'wide appositions' between nerves and odontoblast processes, but these junctions are unlike any junction that could be considered synapse-like. Close examination of similar axon–odontoblastic contacts recognised in cats also indicates that these are non-synaptic. In the present study, however, synapse-like structures comparable to the normal axo-dendritic chemical synapse were found. Numerous EM studies have shown that synapses of vertebrates possess the following basic similarities: discontinuity between the cytoplasm of the two apposed membranes of a synapse, direct contact of the presynaptic and postsynaptic membranes, which are separated by a minute synaptic cleft, usually 10 to 20 nm in width, the presence of mitochondria, neurofilaments and numerous presynaptic vesicles. All of the examined synapses in the database of the present study have these characteristics.

At the light-microscopic level it is still unclear whether the presynaptic vesicles are only present in the neurites that surround the odontoblast or if the odontoblast itself also contains these vesicles. EM analysis, however, showed the presence of vesicles in both the neurites and the odontoblast. Immuno-EM is still necessary to confirm the nature of the vesicles present in the odontoblast processes.

All immuno-stainings contributed to the detection of neuro-odontoblast interactions. As it has previously been described that HGF has the ability to attract sensory nerve fibres[18], the present results led to the hypothesis that odontoblasts could use HGF to establish and to support the neuro-odontoblast interactions seen in dental pulp.

Another argument supporting the neuro–odontoblast interactions is the presence of cilia on odontoblasts and the intimate relationship of these cilia with neural varicosities, suggesting a mechanosensory function. Our findings on the cilia-neural interactions are in accordance with the hypothesis of Magloire et al[14].

For many years the cause of dentine sensitivity has been explained by the movement of tubular fluid, which could excite pulpal mechanoreceptors. The cilia and their intimate relation to neural varicosities as described in the present study might be involved in mechano- or chemo-perception. Neuro-odontoblast synapses are present in teeth and may contribute to dentine sensitivity.

Acknowledgements

The authors would like to thank M. Jans, M-J. Sleypen and J. Hahner for excellent technical assistance in performing the immunohistochemistry.

References

1. Frank RM. Electron microscopic study on odontoblasts and canaliculi in human dentin. Arch Oral Biol 1966;11:179-199.
2. Hargreaves K, Goodis H (eds). Seltzer's and Bender's Dental Pulp, 3rd edition. Chicago: Quintessence, 2002.
3. Frank RM, Sauvage C, Frank P. Morphological basis of dental sensitivity. Int Dent J 1972;22:1-19.
4. Arwill T, Edwall L, Lilja J, Olgart L, Svensson SE. Ultrastructure of nerves in the dentinal-pulp border zone after sensory and autonomic nerve transection in the cat. Acta Odontol Scand 1973;31:273-281.
5. Corpron RE, Avery JK, Lee SD. Ultrastructure of capillaries in the odontoblastic layer. J Dent Res 1973;52:393.
6. Byers MR, Neuhaus SJ, Gehrig JD. Dental sensory receptor structure in human teeth. Pain 1982;13:221-235.
7. Holland GR, Robinson PP. Reinnervation of the canine tooth pulp after section of the inferior alveolar nerve in the cat. Brain Res 1985; 329:300-303.
8. Byers MR. Dental sensory receptors. Int Rev Neurobiol 1984; 25:39-94.

9. Byers MR. Effects of inflammation on dental sensory nerves and vice versa. Proc Finn Dent Soc 1992;88(Suppl 1):499-506.
10. Byers MR, Schatteman GC, Bothwell M. Multiple functions for NGF receptor in developing, aging and injured rat teeth are suggested by epithelial, mesenchymal and neural immunoreactivity. Development 1990;109:461-471.
11. Byers MR, Suzuki H, Maeda T. Dental neuroplasticity, neuro-pulpal interactions, and nerve regeneration. Microsc Res Tech 2003;60:503-515.
12. Tabata MJ, Kim K, Liu JG et al. Hepatocyte growth factor is involved in the morphogenesis of tooth germ in murine molars. Development 1996;122:1243-1251.
13. Kajihara T, Ohnishi T, Arakaki N, Semba I, Daikuhara Y. Expression of hepatocyte growth factor/scatter factor and c-Met in human dental papilla and fibroblasts from dental papilla. Arch Oral Biol 1999;44:135-147.
14. Magloire H, Couble ML, Romeas A, Bleicher F. Odontoblast primary cilia: facts and hypotheses. Cell Biol Int 2004;28:93-99.
15. Fearnhead RW. The neurohistology of human dentine. Proc R Soc Med 1961;54:877-884.
16. Frank RM. Attachment sites between the odontoblast process and the intradentinal nerve fibre. Arch Oral Biol 1968;13:833-834.
17. Byers MR, Kish SJ. Delineation of somatic nerve endings in rat teeth by radioautography of axon-transported protein. J Dent Res 1976;55:419-425.
18. Maina F, Hilton MC, Ponzetto C, Davies AM, Klein R. Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. Genes Dev 1997;11:3341-3350.