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Evidence for changing nerve growth factor signalling mechanisms during development, maturation and ageing in the rat molar pulp

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Abstract

Aim To examine rat molar pulp innervation and identify complex cellular signalling systems involving nerve growth factor (NGF) and its p^{75} receptors (NGFR) at different stages of development, maturation and ageing.

Methodology Decalcified mandibular first molar mesial cusps from Wistar rats of ages 0 day; 2,3,4,6,9,12, and 24 weeks (n=5 per group) were sectioned (10 µm) and incubated with antibodies for NGF, NGFR, calcitonin gene related peptide (CGRP) and neurofilament. Nerve densities in worn and intact regions for age 3-24 week rats were compared by ANOVA, Bonferroni and T-tests.

Results During odontogenesis, differences in NGF and NGFR expression were observed, with no evidence of nerve fibres, suggesting a signalling mechanism controlling cellular differentiation and dentine formation. Tooth wear in 4 week rats was associated with reduced NGF expression and significantly decreased CGRP axons within affected odontoblast regions. The underlying subodontoblasts started expressing NGF which continued until 9w. This may promote a significant increase in CGRP nerve density in affected regions. Nerve density in intact odontoblast regions increased gradually and reached significant levels in 12 week rats. Reduction in nerve densities within worn and intact regions of cusps was observed at 24 weeks.

Conclusions Age-related changes and responses to tooth wear may be controlled by the NGF signalling mechanism, with roles in odontoblast/subodontoblast communication and control of sensory innervation at different stages of tooth development, maturation and ageing. Greater understanding of cellular and nerve regulation in the injured pulp may promote therapeutic strategies for pulp survival.

Introduction

Changes in cell morphology, behaviour and function are observed during the life cycle of the odontoblast cells, which are crucial to maintain their vitality (Couve 1986). These changes are controlled by specific autocrine and paracrine signalling mechanisms such as growth factors, to modulate physiological and patho-physiological changes (Smith & Lesot 2001). Nerve growth factor (NGF) is a member of the neurotrophin family, promoting neuron development, maintenance and repair (Chao 2003). Additionally, it has been reported to be involved in the epithelial-meshenchymal induction associated with tooth development, by its action on p^{75} neurotrophin receptors (NGFR) (Mitsiadis *et al.* 1992). It also presents in mature pulp tissue, with suggested involvement in pulp inflammatory reaction and repair after trauma (Byers *et al.* 1992). However, its role in tooth development, control of innervation and tissue repair after physiological tooth wear is not well known.

Dental pulp, particularly the cusp region, is highly-innervated. Much of this is believed to be sensory innervation, predominantly nociception (Abd-Elmeguid & Yu 2009). In normal conditions, there is no nerve stimulation above nociceptive threshold, (Abd-Elmeguid & Yu 2009), but in pathological conditions, these nerves become hypersensitive to simple thermal, mechanical, and osmotic stimuli, resulting in the sensation of pain, which may become severe (Byers & Narhi 1999). This stimulation has been found in short-term experimental models to be reversible (Taylor & Byers 1990, Taylor *et al.* 1988, Zhang & Fukuyama 1999). None of the previous research has explored the effects of long term trauma such as physiological tooth wear on pulp innervation.

It has recently been suggested that cytoskeletal structural changes occur during development, maturation, ageing, trauma and repair within rat molar and incisor samples (Mahdee *et al.* 2018). However, the cellular signalling mechanisms that control these changes are not well understood. The current work seeks to explore the role of NGF and NGFR by identifying changes in their

expression from early cellular differentiation in the pulp, through primary dentinogenesis, tooth eruption, wear, maturation and ageing in the rat first molar mesial cusp. Observations are linked with changes in pulp innervation, both morphologically and quantitatively, during these different stages of life.

Materials and Methods

All animal procedures were conducted according to Schedule 1, UK Home Office guidelines (UK Home Office 2014). Male Wistar rats of different ages: zero day (0d), 1, 2, 3, 4, 6, 9,12 and 24 weeks (w) (n= 5 to 6 in each) were included. After killing in a CO_2 chamber, mandibles were carefully dissected and divided centrally into two halves. Each half was sectioned into three, with middle pieces containing the 3 molars included in this experiment. Investigation focussed on the mesial cusp of first molars. Samples were fixed immediately in freshly prepared 4% paraformaldehyde solution for 24h before washing thoroughly in phosphate buffer saline. Zero day and 1w samples were examined without demineralisation. All others were demineralised in 17% EDTA (pH 7.4) before preparing 10 µm frozen sections. Twenty to thirty slides were obtained from each block and stored at -80º C.

Slides were processed for immunohistochemical staining as described by Mahdee *et al.* (2016). This included staining for one or combinations of two of the following: rabbit polyclonal anti nerve growth factor (NGF) (1:500, cat # sc-548, Santa Cruz Biotech), goat polyclonal anti nerve growth factor receptor p75 (NGFR) (1:100, cat # sc-6188, Santa Cruz Biotech), mouse monoclonal anti-calcitonin gene related peptide (CGRP) (1:500, cat # sc-57053, Santa Cruz Biotech, Heidelberg, Germany), and rabbit monoclonal anti neurofilament heavy (Nf) (1:1000, cat # Ab40796, Abcam). Negative controls included either isotype controls or incubation of slides with phosphate buffer saline only. Isotype controls were as follows: rabbit IgG monoclonal (EPR25A) isotype control (1:500, cat # ab172730, Abcam, Cambridge, UK) and normal mouse IgG1 (1:500, cat # sc-3877, Santa Cruz Biotech). Stained slides were examined as previously described (Mahdee *et al.* 2016).

In sections stained for CGRP, nerve density was measured within the odontoblast layer of the mesial cusp in animals of 3, 4, 6, 9, 12 and 24w. The region for nerve counting was determined within 200 μm of the cusp margin of the pulp. Nerve counts were made within defined areas of the odontoblast region. In order to collect data from different regions and samples, nerve density was calculated for each measurement and expressed as the number of nerves/ 1000 μm^2. Because tooth wear was identified toward the distal side of the mesial cusp, the nerve density within each cusp was measured separately on each side. Therefore, the worn side nerve density (WND) represent distal side and intact side nerve density (IND) represent the mesial side of the mesial cusp. Whole cusp nerve density (CND) represents the total WND and IND. Measurements were made on one representative slide from each rat (n=5 to 6) in each age group and analyzed by ANOVA and Bonferroni *post-hoc* test to compare WND, IND, and CND between different age groups. Unpaired T-tests were also used to compare WND and IND within the same age group.

Results

All figures show the mesial side (intact side) of the first molar mesial cusp on the left side.

NGF and NGFR

In 0d sections, NGF and NGFR immunoreactivity (IR) varied according to the degree of cellular differentiation (Figure 1 a). In regions of the tooth germ, where the peripheral dental papilla (DP) cells are soon to differentiate into Od, NGF-IR is expressed in the cytoplasm, along with faint staining for NGFR. Adjacent dental papilla cells also express faint NGFR-IR, while inner enamel epithelium cells (IEE) have no evident immunoreactivity to any antibodies. In regions of the tooth germ that are still in the cellular division stage and not actively differentiating, including the inner enamel epithelium cells (IEE) and central region of the dental papilla (lower portion of the tooth germ in Figure 1 a), cells show NGFR-IR. However, the peripheral dental papilla cells (UOd) show no NGFR-IR.

After the start of dentinogenesis, NGF-IR appears more intense in the odontoblasts (Od), especially within the apical part of their cell bodies and the basal part of their processes. NGFR-IR is evident within subodontoblast (SOd) and adjacent central pulp cells (CPC) of the cusp region (Figure 1 b). In 3w specimens, after tooth eruption, odontoblasts express a greater intensity of NGF-IR (Figure 1 c). NGFR-IR is also more intense in cells of the subodontoblast and adjacent central pulp cells. However, the immunoreactivity for both NGF and NGFR reduces gradually toward the cervical line and disappears in the root portion of the tooth.

When cusp wear becomes apparent in 4w specimens, further changes in NGF and NGFR expression are observed, most strongly toward the distal side of the cusp. Both affected odontoblast and adjacent subodontoblast cells show reduced NGF and NGFR-IR respectively (Figure 1 e and d), while some nerves within the subodontoblast region are NGFR-IR. At the same time, subodontoblast cells show faint NGF-IR. In older samples (6-9w), both odontoblast and subodontoblast cells express NGF-IR (arrow in Figure 1 f, f1-2), in addition to apparent NGFR-IR within odontoblast cells on both sides of the cusp (Figure 1 f2).

In 12w sections, odontoblast and subodontoblast cells in regions affected by wear show the return of NGF and NGFR-IR respectively, similar to other intact regions of the cusp (Figure 1 g). Additionally, several nerves, either single or within nerve bundles, show NGFR-IR. This expression remains in 24w samples, excluding pulp regions beneath areas of dentine newly exposed by progressive wear (Figure 1 h (×2)). These regions show no evidence of either NGF or NGFR within affected pulp cells.

Sensory nerve markers (CGRP and Nf)

During early tooth development, only a few pioneer axons enter the dental papilla during crown morphogenesis (Figure 2 a). The number of nerves, especially CGRP-IR fibres, increases in 2w sections when coronal dentine deposition is nearly complete (Figure 2 b and c). A huge increase in

the number of both CGRP and Nf-IR nerve fibres occurs after tooth eruption in 3w sections (Figure 2 d-g). The CGRP-IR axons can be easily distinguished by their varicosities which represent CGRP secretory vesicles. By contrast, the Nf-IR axons appear more uniform without obvious varicosities. Nerves run within a main bundle in the central region of the cusp, in association with large blood vessels which form a neurovascular bundle (Figure 2 e). Major branches leave this bundle with mean diameters of 1.3 μm for CGRP-IR and 1.5 μm for Nf-IR branches. These also give minor branches (0.63 μm for CGRP-IR and 0.69 μm for Nf-IR) which run within the subodontoblast region parallel to the odontoblast cell layer and perpendicular to the long axis of the odontoblast cells. Single CGRP-IR axons (0.38 μm) appear to merge between odontoblast cells into the predentine region. This means that the CGRP-IR branches described above, which come off the main neurovascular bundle, may contain between 10- 20 single CGRP axons. The Nf-IR axons are mainly limited within the subodontoblast region, with very few nerves observed within the odontoblast layer especially in the pulp horn region (Figure 2 f). Since root formation continues during this period, nerve development is only limited within the coronal part of the pulp, occlusal to the cervical line (Figure 2 g).

Dentine exposure due to wear, which is apparent in 4w sections, is also associated with changes in nerve fibre distribution within affected regions of the pulp (Figure 2 h). No evidence of CGRP-IR and Nf-IR axons is recognised between odontoblast cells under worn region. Some nuclei of detached pulp cells are also recognised within reactionary dentine. However, the intact mesial side of the cusp shows an increase in the number of CGRP-IR fibres within odontoblast layer and emerging into predentine. The fine CGRP axons are observed either inter-cellularly between odontoblasts or in combination with small capillaries (Figure 3 e). Major nerves present within the central pup region run perivascularly. Additionally, nerve development within the pulp region between cusps (groove area) become more apparent at this age than in younger specimens (Figure 2 j). This development is only limited within the coronal region of the tooth, while no evidence of CGRP-IR or Nf-IR fibres is recognised within furcation region of the pulp.

CGRP-IR nerve proliferation and sprouting beneath worn dentine is apparent in 6w specimens, especially in pulp region affected by wear (Figure 3 a and a1). Additionally, the number Nf-IR axons is also increased among adjacent subodontoblast cells. The main neurovascular bundle is clearly identified in Figure 3 (a). From this bundle, numerous major branches arise and run peripherally towards the subodontoblast region, where they arborise into many small minor branches. These branches form a network of nerve fibres that innervate the whole cusp odontoblast layer; the 'subodontoblast nerve plexus', which appears limited to the coronal region of the pulp. A similar pattern of innervation is recognised within older pulp specimens (9 and 12w sections) where a more developed submandibular nerve plexus is also identified (Figure 3 b, b1, and c, c1 respectively). Additionally, there is an increase in nerve fibre numbers within odontoblast regions affected by wear. However, progressive wear exposes new dentinal tubules in 24w sections, and this causes the disappearance of CGRP-IR axons between newly affected odontoblast (Figure 3 d, d1). Signs of CGRP-IR nerve sprouting is also evident within these sites (Figure 3 d1 (arrows)).

None of the negative controls, showed staining in the targeted regions.

CGRP nerve counting

Figure 3 (f) shows numerical values for CGRP nerve densities within the entire mesial cusps (cusp nerve density: CND) and the sub-regions with no damage (intact nerve density: IND) and regions with tooth wear (worn nerve density: WND) at different ages (3 to 24w). Examination of the whole cusp suggest a progressive increasing nerve density from 3 to 12w ($P < 0.001$) and a significant fall in 24w ($P < 0.001$). However, a more detailed examination of the intact and worn regions reveals clear differences, specifically in the worn region. These regions appear to be significantly increased in nerve density at 6w ($P < 0.001$), with no significant change in intact regions. The significant rise seeing overall in (CND) is over entirely due the rise in the nerve density in the worn region. It is quite clear that at 9w the nerve density in the worn region is increased markedly ($P < 0.001$). This suggests

that damage accelerates nerve growth. However, by 12w the intact region of the tooth (IND) has been able to develop the same nerve density as the worn region ($P < 0.001$). Interestingly, beyond 12w, at 24w, the nerve density in both intact and worn regions was observed to decrease significantly ($P < 0.001$). T-tests also revealed significant differences between the IND and WND in 3w ($P < 0.05$), and 4 and 9w groups ($P < 0.001$).

Discussion

NGF with its p^{75} receptors forms part of the cellular signalling mechanisms during tooth development, ageing and trauma (Byers *et al.* 1990, 1992), and play a primary role in regulating nerve fibre growth and sprouting during physiological and patho-physiological conditions (Byers *et al.* 1992, Mitsiadis *et al.* 1992). This study explored NGF and p^{75} receptors expression in rat molars of different ages to identify the possible role for such signalling mechanism during different stages of tooth development, maturation and in response to tooth wear. The immunohistochemical techniques are well established in our previous work (Mahdee *et al.* 2016, 2018), with antibodies thoroughly optimised. However, no quantitative immunohistochemical methods were employed, because of tissue complexity and regional variations within the pulp which impair analysis accuracy. Correlations were also made with the study of nerve markers CGRP and Nf and quantitative comparison of CGRP-IR nerves under intact and worn dentine surfaces. Schematic illustrations that summarise the observations of this study are presented in Figure 4.

Changes in the expression of both NGF and NGFR were observed during the morpho and cyto-differentiation stages of tooth development (Figure 4 a). This corresponds with previous studies, which suggest sequential regulatory signalling between epithelial and mesenchymal elements. This may mediate cellular timing and differentiation during early stages of tooth morphogenesis (Byers *et al.* 1990, Mitsiadis *et al.* 1992). No sensory nerve fibres were evident in similar age group sections. The autonomous, nerve independent, mechanism of NGF synthesis within developing tissue was

previously reported (Rohrer *et al.* 1988). This indicated the ability of the developing dental cells to produce NGF and provide its membrane receptors, suggesting an autocrine or paracrine mode of action (Mitsiadis *et al.* 1992). The presence of this signalling mechanism at this stage of tooth development suggests its active involvement in the cyto-differentiation of the odontoblasts and other cells of the pulp.

In common with previous studies (Byers *et al.* 1990, Luukko *et al.* 1996, Mitsiadis *et al.* 1992) immunoreactivity for NGF and NGFR in odontoblast and subodontoblast cells was also identified during primary dentinogenesis (Figure 4, b and c).. This suggests a 'maestro' role of odontoblasts in controlling pulp cells during primary dentinogenesis and in maturing tissue. This could be related to several spatial and functional properties of the odontoblast layer including their:

1. Formative role in the deposition of tubular dentine and control of its mineralisation before and after tooth eruption (Linde & Goldberg 1993).
2. Derivation from neural crest cells (Ruch *et al.* 1995).
3. Formation of a special cellular barrier, separating pulp from mineralised dentine and/or oral cavity, with cells in this layer linked by numerous gap junctions (Turner *et al.* 1989).

NGF also serves as a neurotrophic factor in regulating and maintaining nerve cells. This induces NGF-responsive neurons growth toward the NGF source (Chao 2003). The presence of NGFR-IR within nerve bundles in the central pulp and small fibres of the subodontoblast region. This could explain the oriented nerve development toward the odontoblast layer and dentine which became apparent after tooth eruption. Therefore, the unique properties of the odontoblast layer with their specific expression of NGF could suggest their control of both pulp cells and nerve supply.

It is unclear from the current study whether the CGRP-IR or Nf-IR fibres within odontoblast and subodontoblast regions were myelinated. However, and depending on the calculated diameters, CGRP-IR fibres were smaller than Nf-IR fibres and contained varicosities. Mainly unmyelinated nerve fibres which contained microvesicles, were reported previously within odontoblast regions and inner dentine, while both myelinated and unmyelinated nerves were observed within subodontoblast region (Corpron & Avery 1973). Such unmyelinated axons are usually sensory A delta or C fibres (Byers 1984, Fristad *et al.* 1994) distinguished to be responsive to CGRP depending on their secretory microvesicles (Taylor & Byers 1990, Taylor *et al.* 1988). An additional type of axon (Nf axons), were located in the current study between subodontoblast cells, and rarely between odontoblasts (smaller diameter). Neurofilament is part of the structural skeleton of the nerve axons (Tsuzuki & Kitamura 1991), and considered as a marker for the myelinated sensory fibres (Luthman *et al.* 1992).

The exposure of dentine by physiological tooth wear was associated with changes in cellular expression of NGF and NGFR (Figure 4 d). The marked reduction in NGF-IR within affected odontoblasts was associated with its appearance within underlying subodontoblast cells. Additionally, there was an increase in its expression in the adjacent intact odontoblasts. These 'injury responses' agreed with previous studies, which reported similar cellular reactions up to 9 days after trauma (Byers *et al.* 1992, Woodnutt *et al.* 2000). This could be part of the pulp inflammatory process within the worn rat molar to support the injured cells preparing for repair. These cellular responses have previously been identified in normal and denervated teeth, indicating a nerve-independent process (Byers *et al.* 1992). The reduction in NGF production by odontoblasts beneath the worn dentine, may stimulate its formation by the underlying subodontoblast and the adjacent non-injured odontoblasts, to compensate for that lost within injured tissues. The lack of NGF mRNA within injured odontoblasts after cavity preparation into dentine has also been reported, suggesting that NGF was transferred to these cells from adjacent subodontoblastic cells (Woodnutt *et al.* 2000). Unpublished data showed different structural alterations within injured odontoblast and

subodontoblast cells following cusp wear in rats. The presence of NGF producing subodontoblast cells within this region could help to mediate and regulate the formation of reactionary dentinogenesis (Magloire *et al.* 2001, Mahdee *et al.* 2016). In other words, the subodontoblastic fibroblasts may control odontoblast function during period of tissue injury (Byers *et al.* 1992, Magloire *et al.* 2001).

The effects of dentine exposure on CGRP-IR nerves was apparent both morphologically and quantitatively. Localised depletion of CGRP-IR fibres beneath areas of dentine exposure appeared similar to previous observations (Taylor & Byers 1990, Taylor *et al.* 1988). The exact mechanism controlling this transient axon retraction is unknown, but it could be part of inflammatory changes resulting from tissue insult. Additionally, reduced expression of NGF-IR within odontoblasts and its evidence within underlying subodontoblast cells could be themediator for this retraction (Figure 4 d).

NGF was reported to induce the invasion of leukocytes to the injured region by its non-neuronal paracrine receptors during inflammation (Woodnutt *et al.* 2000). It may also induce significant morphological changes within odontoblast cells, enhance production of cellular microfilaments within odontoblasts and their processes, activate the nuclear transcription factor (NF-kB), induce formation of trk-A (Woodnutt *et al.* 2000), and p75 NGF receptors within normal and injured odontoblasts (Magloire *et al.* 2001). The presence of this signalling system between adjacent cells possibly controls cellular inflammatory responses and tissue repair mechanisms. Furthermore, the subodontoblast cells continue to express NGF-IR in 6 and 9w specimens (Figure 4 e), which means 5-6w after the onset of dentine exposure. This may help in tissue repair and may also explain the hyper-innervation observed within the worn cusp between 6-12w. This may be mediated by the profound presence of NGF forming cells (Chao 2003). CGRP-IR nerves are assumed to be primarily sensory as is assumed from the physiology of C fibres (Abd-Elmeguid & Yu 2009), and their origin in

the trigeminal ganglia (Pan *et al.* 2003). The observed CGRP nerve proliferation possibly increases nociception in the inflamed pulp. Alternatively, it could increase the release of CGRP and substance P, modulating tissue repair (Taylor & Byers 1990). Additionally, the structural re-establishment of the odontoblast and their morphological changes from inflammatory to reparative patterns could also be mediated by CGRP.

At 12w both IND and WND reached their maximum levels (Figure 4 f). At this age, the apical foramen of the roots of the first molar appeared to be complete and the tooth to be mature. It has been reported that the number of axons increases during tooth maturation and reach its maximum after complete root formation (Byers 1984). Therefore, the increasing number of nerves entering the tooth could explain the increasing axon arborisation within both sides of the mesial cusp. Additionally, the subodontoblast nerve plexus became more developed by this age. However, the WND was still higher than the IND. This could reflect the effect of neurogenic inflammation which could extend to this age.

The involvement of the p^{75} NGF receptors in cellular apoptosis has also been reported (Casaccia-Bonnefil *et al.* 1998). The expression of p^{75} NGFR was seen in the odontoblasts of the rat incisor, whereas its expression was limited to the subodontoblast during primary dentinogenesis (Mitsiadis *et al.* 1993). This could keep the density of the odontoblast cells constant in continuously growing teeth by apoptosis of NGFR expressed odontoblasts (Mitsiadis *et al.* 2008). This pattern of NGFR expression was also evident in this study, with expression only in subodontoblast cells before tooth eruption. After tooth eruption, these receptors became apparent both within odontoblast and subodontoblast cells, especially in older samples (9- 24w) (Figure 4). This could be the mechanism by which cellular apoptosis is controlled during ageing to accommodate the restriction of the total pulp space due to secondary and tertiary dentinogenesis (Lovschall *et al.* 2002, Murray *et al.* 2002). This could also explain the reduction in mesial and distal nerve densities in 24w compared with 12w

specimens. Moreover, the distal nerve density showed more significant changes than the mesial. This could be due to the appearance of new regions of dentine exposure on the distal side of the cusp which showed disappearance of CGRP axons from regions of newly uncovered Od. On the basis of the current data, the consequences of this new trauma site on the CGRP nerve sprouting is unknown. However, previous research reported higher CGRP nerve sprouting after cavity preparation in older compared to younger rats (Swift & Byers 1992).

Conclusions

Changes in the expression of NGF and NGFR in pulp cells during different stages of tooth development, maturation, and ageing suggest the presence of multiple functions indicative of different and discrete signalling operating between the cells of the pulp. This signalling pathway could play pivotal roles during cellular differentiation, dentine formation, defensive responses and tissue repair and regeneration. These functions would be in addition to the more classical role of NGF in controlling nerve distribution, growth and sprouting, especially CGRP-IR fibres, during physiological and patho-physiological conditions. Therefore, a better understanding of these cellular signalling mechanisms could eventually improve our understanding of the processes that occur in human pulp after injury and in therapeutic interventions to optimise vital pulp responses.

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Conflict of interest

The authors state explicitly that there is no conflict of interest in connection with this article.

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Figure Legends

Figure 1 Sagittal sections of rat mandibular first molar mesial cusps of different ages showing the expression of NGF and NGFR. Coloured images are stained for NGF (red), NGFR (green), and dapi (blue), (d, f1) for NGF, and (e, f2) for NGFR. Panel (a) is 0day section, showing the mesial side of the tooth in advanced bell stage, with outer enamel epithelium (OEE), inner enamel epithelium (IEE), undifferentiated odontoblast (UOd), pre-odontoblast (POd), and dental papilla cells (DP). Crown formation (2w section) and tooth eruption (3w section) are seen in (b) and (c) respectively, with evident NGF-IR within the odontoblast layer (Od) and NGFR-IR in subodontoblast (SOd) and central pulp cell (CPC) regions, and a large blood vessel (BV). Panels (d) and (e) are 4w sections showing early cellular responses to cuspal wear (×).and NGFR-IR axons within the subodontoblast region (arrows in e). A 6w section (f) and its component images (f1) and (f2), show persistent NGF-IR within the subodontoblast cells (arrow). Panels (g) and (h) are 12w and 24w sections respectively. A new region of dentine exposure (×2) has appeared more distal to the initial region of wear (×1). Scale bar = 200 µm panels (a), (b) and (g); = 100 µm in remaining panels.

Figure 2 The expression of CGRP and Nf nerve markers within sagittal sections of the mesial cusps of 1- 4w rat mandibular first molars. Coloured images stained for CGRP (green), Nf (red) and dapi (blue), (b) for CGRP and (c) for Nf. Panel (a), 1w section showing: odontoblasts (Od), subodontoblast cells (SOd), central pulp cells (CPC), dentine (De), blood vessel (BV). Panels (b) and (c) are 2w sections showing more CGRP-IR nerve development within mesial cusp. Panel (d) 3w section, showing increased numbers of CGRP and Nf-IR nerves in the cuspal region. Three regions of interest are shown at higher magnification in Panels (e) – (g). A double-sided arrow in (g) orientates crown (C) and root (R) directions of the image in the cervical region (dotted line). Panel (h): 4w section showing no evidence of CGRP-IR nerves within odontoblast layer beneath exposed dentine (×), and some nuclei detached from the cell layer (*). More nerve fibres are identified on the mesial side of the cusp (arrow). Panel (i): 4w section, comparing pulp innervation between groove (upward) and

furcation (downward) regions. Scale bar = 200 μm in (d and j), 100 μm in (c) and (h), 75 μm in (a) and (b), 25 μm in (e), (f) and (g).

Figure 3 Innervation and nerve densities in the mesial cusp pulp of rat mandibular first molars of different ages. Sections stained for CGRP (green), Nf (red) and dapi (blue), showing: odontoblasts (Od), subodontoblast cells (SOd), blood vessel (BV). Panels (a, a1) for 6w, (b, b1) for 9w, (c, c1) for 12w, and (d, d1) for 24w samples. Panels (a1) – (d1) are higher magnification images of the pulp region responding to worn cusp surface (×, ×1-2) for images (a) – (d) respectively. Panels (d) and (d1) show a new pulp response (×2) to progressive wear. Panel (e): 6w section showing one nerve wrapping around an odontoblast (arrow) and another wrapping around a capillary (*). Panel (f) is bar chart includes intact-side nerve density (IND), worn-side nerve density (WND) and whole mesial cusp nerve density (CND) (nerves/ 1000 μm^2) of different age groups Scale bar = 100 μm in (a) – (d), 50 μm in (a1) – (d1), and 20 μm in (e).

Figure 4 Schematic representation of NGF, NGFR-IR and CGRP nerve distribution in all study groups with predentine (PD), pre-odontoblast (POd), inner enamel epithelium (IEE), undifferentiated odontoblast (UOd), outer enamel epithelium (OEE), dental papilla (DP), odontoblast (Od), subodontoblast (SOd), central pulp cells (CPC), enamel (En), dentine (De), blood vessel (BV), worn dentine pulp response region (×, ×1, ×2), neurovascular bundle (▲, major branch (*), minor branch (+), and single CGRP axon (●).