*Original Article*

# Changes in the Distribution of Nerve Fibers Immunoreactive to Calcitonin Gene-Related Peptide According to Growth and Aging in Rat Molar Periodontal Ligament

**Hangsul Jung[a]; Mariko Horiuchi[b]; Kunimichi Soma[c]**

**ABSTRACT**

**Objective:** To analyze the age-dependent changes in nerve fibers immunoreactive to calcitonin gene-related peptide (CGRP-ir) in the periodontal ligaments of rats.

**Materials and Methods:** Thirty male Wistar-ST rats were divided into growing groups (5, 9, and 15 weeks of age) and aging groups (6, 12, and 24 months of age) (n = 5 in each group). Eight serial sagittal sections, 5 μm thick, were cut parallel to the distobuccal root of the maxillary right first molar. These tissues were stained with a rabbit monoclonal antibody against CGRP. The observation area was divided into three parts (mesial, apical, and distal) and observed using a light microscope.

**Results:** CGRP-ir nerve fibers were primarily distributed in the apical periodontal ligament in the growing group, with significantly more fibers than in the aging group.

**Conclusions:** CGRP-ir nerve fibers in the periodontal ligament are dense during the growth period and decrease gradually with aging, indicating that CGRP may affect periodontal tissue with growth and aging. (*Angle Orthod.* 2010;80:309–315.)

**KEY WORDS:** Growth and aging; Periodontal ligament; Calcitonin gene-related peptide

## INTRODUCTION

In recent years, orthodontic treatment has increasingly been given to adults, rather than just children or adolescents. This is a result of the extended average life span, increased interest in quality of life and personal appearance on the part of patients, and advances in diagnostic and orthodontic techniques and materials.

The human life process is one of growth, development, and aging. During this process, changes occur in various bodily functions, such as those that confer metabolic efficiency. In the periodontal tissue, structural changes occur, such as narrowing of the periodontal ligament (PDL) and apposition of cementum to the nearby root.[1,2] To provide orthodontic treatment that is best suited to each patient, the diverse biological changes that occur in the PDL with growth and aging require elucidation.

During orthodontic tooth movement, various tissues such as bone, blood vessels, and collagen fibers in periodontal tissues are remodeled; neuropeptides are involved in the remodeling process.[3] The primary neuropeptides that are distributed in the periodontal tissue are calcitonin gene-related peptide (CGRP), substance P, and vasoactive intestinal peptide.[4] These neuropeptides increase during tooth movement[3] and decrease in the nonocclusion that follows tooth replantation in rats.[5] Via these actions, they are involved in the control of blood vessels, immune cells, osteoclasts, osteoblasts, and fibroblasts.

CGRP participates in the inflammatory response as a potent vasodilator to increase vascular permeability, plasma extravasation, and proliferation of endothelial cells and fibroblasts. CGRP also up-regulates the expression of endothelial cell adhesion molecules and

[a] Graduate PhD Student, Orthodontic Science, Department of Orofacial Development and Function, Tokyo Medical and Dental University, Tokyo, Japan.

[b] Assistant Professor, Orthodontic Science, Department of Orofacial Development and Function, Tokyo Medical and Dental University, Tokyo, Japan.

[c] Professor Emeritus, Tokyo Medical and Dental University, Tokyo, Japan.

Corresponding author: Hangsul Jung, DDS, Orthodontic Science, Department of Orofacial Development and Function, Division of Oral Health Sciences, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8549, Japan
(e-mail: sulsul82.orts@tmd.ac.jp)

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accelerates bone formation by inhibiting osteoclasts and increasing osteoblast activity.[3] CGRP expression decreases in the temporomandibular joint from 6 weeks to 9 weeks,[6,7] increases with growth, and decreases with aging in the thyroid gland, veins, and other tissues.[7–11] CGRP expression also decreases in the trigeminal ganglion with age from 3 to 36 months in rats.[12,13]

CGRP is mainly distributed in the root apex area[3] and influences tissue remodeling, but no reports have examined CGRP expression in the PDL with growth and aging. Here, we measured changes in the distribution of nerve fibers immunoreactive to CGRP (CGRP-ir) in periodontal tissue with growth and aging.

## MATERIAL AND METHODS

### Animal and Tissue Preparation

Animal protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University. The experiments were carried out under the control of the University's Guidelines for Animal Experimentation.

Thirty male Wistar-ST rats (Sankyo Lab Service Corporation Inc, Tokyo, Japan) ranging in age from 5 weeks old to 24 months old were used. They were housed three or four per cage, had free access to food and water, and were kept on a diurnal lighting schedule.

At 5, 9, and 15 weeks of age and at 6, 12, and 24 months of age (each group, n = 5), after the administration of diethyl ether for anesthesia, rats were deeply anesthetized via intraperitoneal injection of chloral hydrate (400 mg/kg); this was followed by transcardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The maxillae were excised en bloc and further immersed in the same fixative solution at 4°C for 12 hours. The specimens were rinsed in phosphate-buffered saline, decalcified in 10% ethylenediamine tetraacetic acid disodium solution at 4°C for 7 weeks, and embedded in paraffin by conventional methods. Five-μm-thick serial sagittal sections were cut parallel to the distobuccal root of the maxillary right first molar (M1) (RM 2155, Leica Co LTDA, Nussloch, Germany).[5] These sections included the surrounding tissue (Figure 1A,B).

### Staining

Hematoxylin and eosin staining of the sagittal sections in all rats were made to investigate the effects of growth and aging effects on the tissues. Immunohistochemical staining was performed using a catalyzed signal amplification system (HRP, Dako, Carpinteria, Calif) for CGRP according to the manufacturer's instructions. CGRP polyclonal antibody (Yanaihara Institute, Shizuoka, Japan) was applied at a dilution of 1:3000 for 15 min. The protocol followed was essentially that described by Takei et al.[6]

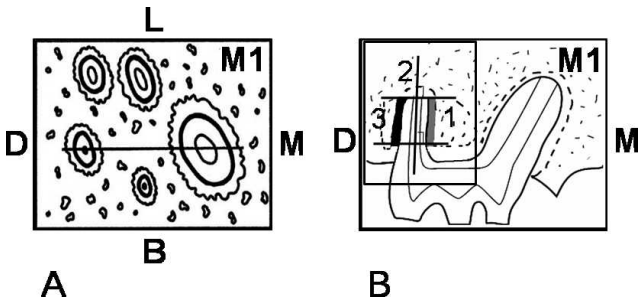


**Figure 1.** (A) Diagram showing a horizontal view of M1. The line indicates the central area of the mesiobuccal root and distobuccal root of M1. (B) Schematic drawing of a sagittal section of M1. The line dividing the distal root longitudinally was drawn, two additional perpendicular lines were drawn 100 μm below the root apex, and the line passing the roof of the alveolar bone was drawn. The observation area was divided into three parts (1 indicates mesial; 2, apical; and 3, distal) by these lines and the periodontal membrane. M1 indicates maxillary first molar; M, mesial, D, distal; B, buccal; and L, lingual.

### Measurements

Eight serial sections of the central area of the mesiobuccal and distobuccal roots of the maxillary M1 were added to quantify the area containing CGRP-ir nerve fibers. The measured area was demarcated by a line dividing the distal root longitudinally and two lines that were drawn perpendicular to the initial longitudinal line: a line passing 100 μm below the root apex and a line passing over the roof of the alveolar bone. The observation area was divided into three parts (mesial, apical, and distal) by these lines and the periodontal membrane (Figure 1B). The stained sections were examined under a light microscope. The measurements of the immunoreactive area and density in the PDL of CGRP-ir nerve fibers were made using Image Pro Plus image analysis software (version 4.0, Media Cybernetics, Md).

### Statistical Analysis

Tukey's highly significant difference test was used for all the statistical analyses in this study to determine significant differences between groups, using statistical analysis software (SPSS 10.0J, Chicago, Ill). Calculated data were normally distributed and expressed as means ± standard deviations.

## RESULTS

### Body Weight

The body weight of the rats increased from 5 weeks to 6 months. There was no significant change in body weight between 6 and 24 months. (Data not shown.)



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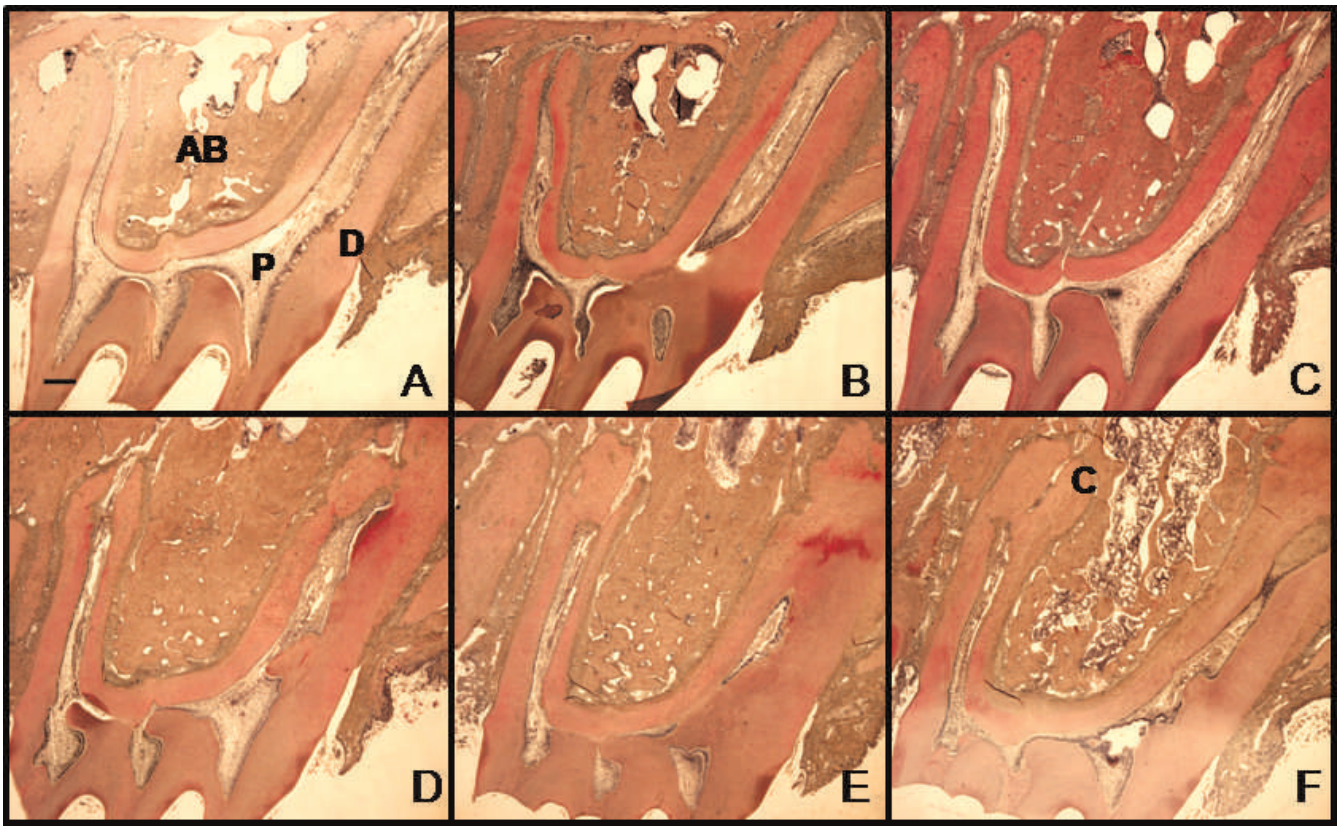


**Figure 2.** Light micrographs of 5-μm sagittal sections of M1 (hematoxylin and eosin staining). (A) Five weeks old. (B) Nine weeks old. (C) Fifteen weeks old. (D) Six months old. (E) Twelve months old. (F) Twenty-four months old. AB indicates alveolar bone; P, pulp; D, dentin; and C, cementum. Bar = 200 μm.

### Morphological Changes in Molar Teeth with Aging

At 5 weeks of age, the root apex is wide open and being formed; the root is relatively short. The PDL is wide and the pulp cavity is large at this age (Figure 2A). Following growth, at 9 and 15 weeks of age, the root has become longer and formation of the root apex is complete (Figure 2B,C). The alveolar bone has become denser. By 6 months of age, the tertiary dentin in the pulp cavity has been formed, but the root apex is still open (Figure 2D). By 12 months, the beginnings of cusp attrition can be observed, the root apex has closed, and the PDL has narrowed, with an increase in cementum (Figure 2E). By 24 months, the bone marrow cavity has increased in size, absorption of the surface of the alveolar bone can be observed, and extrusion of the tooth has become obvious (Figure 2F).

### Distribution of CGRP-ir Nerve Fibers in the PDL

The CGRP-ir nerve fibers were more common in the apical PDL than in the mesial and distal PDL at 5, 9, and 15 weeks and at 6 months ($P < .01$). This difference was not seen at 12 or 24 months (Figure 3; Table 1).

### Histological Observation of CGRP-ir Nerve Fibers

In all groups, CGRP-ir nerve fibers were observed in the PDL, pulp, alveolar bone, and gingiva. Thick nerve bundles present in the apical PDL branched into thin nerve fibers toward the pulp. CGRP-ir nerve fibers were found along the blood vessels, which were detected in the margin of the alveolar bone. Isolated fibers were also identified. There were no significant differences among the groups in this distribution (Figures 3 and 4). More findings of degeneration, such as separation between the fibers of nerve fiber bundles, structures that resembled strings of beads, and a reduction in staining, appeared in the root apical PDL of rats at 12 and 24 months than at 5, 9, 15 weeks and 6 months (Figure 4).

### Quantitative Analysis of CGRP-ir Nerve Fibers in the Apical PDL

The area of CGRP-ir nerve fibers in the apical PDL was larger at 9 and 15 weeks than at 5 weeks or at 6, 12, and 24 months old, but not different at 9 and 15 weeks (Figures 4 and 5A). The density of CGRP-ir nerve fibers in the apical PDL was higher at 15 weeks than at 5 weeks or at 12 and 24 months but not different at 9 weeks, 15 weeks, and 6 months (Figures 4 and 5B).



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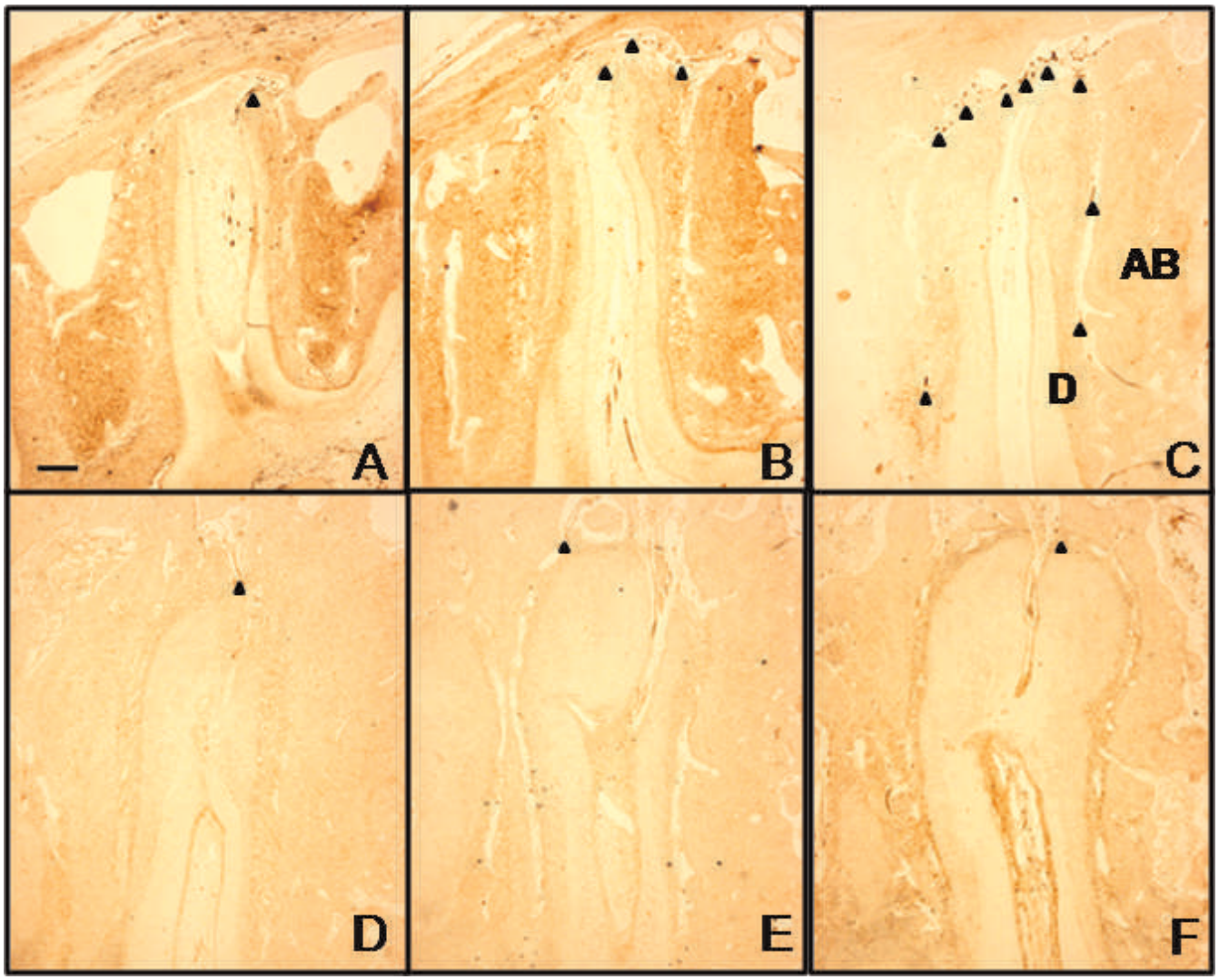


**Figure 3.** Immunostaining of CGRP in the measurement site (areas shown in Figure 1B). Immunopositive nerve fibers are stained with brown (arrowheads). (A) Five weeks old. (B) Nine weeks old. (C) Fifteen weeks old. (D) Six months old. (E) Twelve months old. (F) Twenty-four months old. AB indicates alveolar bone; P, pulp; D, dentin; C, cementum. Bar = 200 μm.

## DISCUSSION

Rats and humans have different life spans, but they can still be compared. These life spans can be divided into nursing, prepubescent, adolescent, adult, and aged phases. In humans, 6 months, 12 years, 20 years, 50 years, and 80 years are used as reference points, with equivalent points in the rat of 9 days, 7 weeks, 6 months, 20 months, and 36 months, respectively.[13] According to this standard, rats at 5 weeks of age correspond to prepubescent humans of about 9 years. Rats at 9 and 15 weeks correspond to adolescent humans of 15 and 18 years, respectively; rats of 6 and 12 months are equivalent to adult humans

**Table 1.** CGRP-ir Nerve Fiber Dimensions in the Measurement Site[a]

| | 5 wk | 9 wk | 15 wk | 6 mo | 12 mo | 24 mo |
|---|---|---|---|---|---|---|
| Mesial | 20.17 ± 11.86 | 18.5 ± 9.66 | 25.48 ± 15.12 | 16.61 ± 5.75 | 5.56 ± 3.72 | 15.06 ± 3.16 |
| Apex | 47.1 ± 9.8 | 105.56 ± 25.21 | 118.46 ± 31.54 | 52.43 ± 5.58 | 29.64 ± 16.57 | 37.92 ± 19.99 |
| Distal | 4.86 ± 4.18 | 14.34 ± 14.2 | 12.28 ± 7.75 | 8.32 ± 2.92 | 10.89 ± 9.03 | 20.42 ± 8.46 |
| Mesial, apex | ** | ** | ** | ** | – | – |
| Apex, distal | ** | ** | ** | ** | – | – |
| Mesial, distal | – | – | – | – | – | – |

[a] Areas (× 100 μm²; means ± standard deviations) shown in Figure 1B; n = 5 for each group. Three areas are compared in each column. Significant differences between the two areas are marked with asterisks (**$P < .01$).



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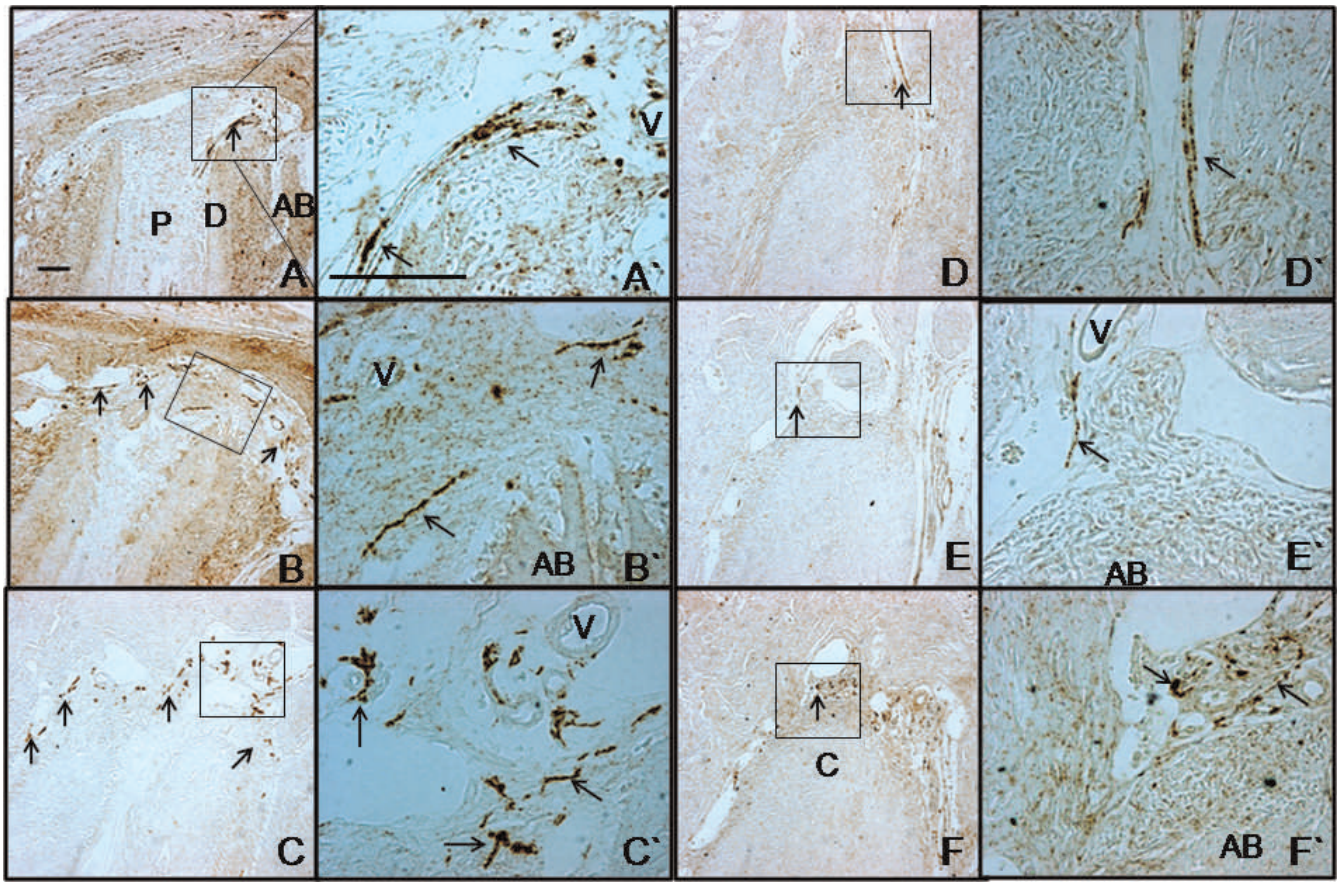


**Figure 4.** Immunostaining of CGRP in the root apex area (areas shown in Figure 1B). Immunopositive nerve fibers are stained with brown (arrowheads). The CGRP-ir nerve fibers in E and F have structures that resemble strings of beads and low-intensity staining. They are also thinner and more truncated than those in A, B, C, and D. (A) Five weeks old. (B) Nine weeks old. (C) Fifteen weeks old. (D) Six months old. (E) Twelve months old. (F) Twenty-four months old. AB indicates alveolar bone; P, pulp; D, dentin; C, cementum; V, vessel. Bar = 100 μm.

of 20 and 35 years, respectively, and those of 24 months correspond to humans of 60 years.

With aging, the width of the PDL decreases as a result of cementum formation, but the number of cementum granules in the PDL increases. The surface of the cementum and alveolar bone is no longer smooth and assumes a scalloped appearance.[14] We also found a narrower PDL (Figure 2E), increased cementum deposition (Figure 2D), and absorption of the surface of the alveolar bone (Figure 2F) in aged animals.

The root of the rat molar is half-formed at 3 weeks, two-thirds complete at 6 weeks, and three-quarters

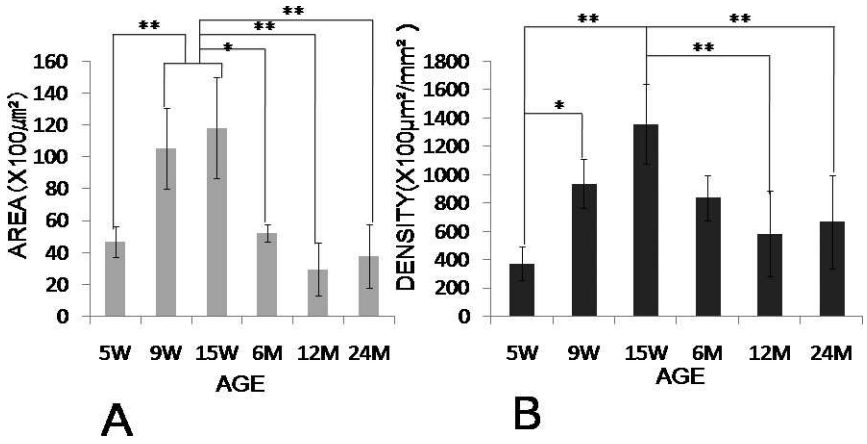


**Figure 5.** CGRP-ir nerve fiber density in the apical PDL (areas shown in Figure 1B); n = 5 for each group. Values are means ± standard deviations. Significant differences between the two groups are marked with asterisks (*$P < .05$, **$P < .01$).



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formed by 8 weeks after birth.[15] The root is fully developed at about 100 to 120 days (14–17 weeks)[16] (Figures 3A through C). In our study, CGRP expression in the apical PDL increased after 5 weeks and decreased again after 6 months (Figures 4 and 5). Rats at 5, 9, and 15 weeks have roots that are progressing from two-thirds formed to completion. The CGRP receptor was found on cells close to the dentin, cementum, and alveolar bone of the developing root.[17] The increased expression of CGRP in the root apex during the growth period (9 to 15 weeks) may occur in response to the requirements for root formation.

Root formation in the rat molar begins 2 weeks after birth, and the tooth is in functional occlusion 3 weeks after the onset of root formation.[18] Occlusal loading is a well-known mechanical modulator of bone remodeling. The number of fibers immunoreactive to vasoactive intestinal peptide increased significantly in an occluded group of rats, compared to a nonoccluded group, following tooth reimplantation.[5] With traumatic occlusion of rat molars, the density of nerves immunoreactive to CGRP and substance P is increased in the local gingiva, PDL, and pulp.[19,20] Loading by occlusal force causes an increase in the number of nerve fibers positive for neurofilament protein, many of which have free nerve endings in the peri-implant tissue.[21] Increased CGRP expression in the apical PDL after 5 weeks may be related to occlusal mechanical stress.

Maxillary molar neurons connect to maxillary nerves that lead into the trigeminal ganglion. The number and fluorescence intensity of CGRP-ir nerve fibers in the trigeminal ganglion decrease in aged rats (36 months old)[12,22]; this is consistent with our findings of lower CGRP levels in 24-month-old rats than in 9- and 15-week-old rats (Figures 4 and 5). This may be related to reduction that happens in the root apex, but to date, no studies have examined CGRP-ir nerve fibers in the trigeminal ganglion during growth.

Although the magnitude of changes in CGRP-ir density was smaller than changes in area (Fig. 5A,B), probably because of the decreased width of the PDL with growth and aging, both measurements showed the same overall trends.

The number of nerve fibers in the PDL increases while roots develop during formation of the apex, with the highest density in the apex, followed by the alveolar crest, bifurcation, and middle of the root. Between 6 and 24 months, signs of degeneration included separation between nerve fibers, fiber swelling, and reduced staining.[1,23,24] We found similar changes in distribution and morphology in CGRP-ir nerve fibers (Figures 3 and 4; Table 1), but there were larger decreases in the aged groups. Therefore, aging may both reduce general fiber density and CGRP production.

CGRP increases collagen synthesis through fibroblast proliferation and increases bone formation by activating osteoblasts and inhibiting osteoclasts.[3] Such effects may act unfavorably in orthodontic tooth movement. The movement of teeth in orthodontic treatment may potentially be slower in adults than in children because of CGRP activity.

## CONCLUSIONS

- CGRP-ir nerve fibers in the PDL are densest during growth and gradually decrease with aging.
- CGRP may influence changes in periodontal tissue with growth and aging.

## ACKNOWLEDGMENT

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