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# Identification of Thyrotropin-Releasing Hormone (TRH)-Degrading Enzyme as a Biomarker for Dental Pulp Tissue

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## Abstract

Regeneration of dental pulp after pulpectomy was accomplished recently by autologous transplantation of dental pulp stem/progenitor cells into the root canal. The identical patterns of qualitative and quantitative protein and mRNA expression in the regenerated pulp and normal pulp demonstrated complete pulp regeneration. The lack of tissue specific markers in the dental pulp is a major challenge in dental regenerative medicine. In order to identify specific markers in dental pulp we undertook a comparison of the gene expression profile of the dental pulp with that of periodontal ligament and gingiva. This systematic investigation identified thyrotropin-releasing hormone (TRH)-degrading enzyme (DE) as a marker of dental pulp. Expression of TRH-DE mRNA in human dental pulp was higher than that in any other tissue except brain as analyzed by real time RT-PCR. Induction of neural cells enhanced the expression of TRH-DE mRNA in dental pulp stem/progenitor cells (CD105+ and CD31- side population (SP) cells) in vitro. Immunohistochemical and in situ hybridization analyses demonstrated that TRH-DE in the neuronal processes in dental pulp. In canine pulp cells, TRH down-regulated TRH-DE mRNA expression, while neuropeptide Y up-regulated it, suggesting that TRH-DE has functional role in the neuropeptide signaling in dental pulp tissue. It is noteworthy that TRH-DE mRNA was expressed in the regenerated pulp 28 days after transplantation of CD31- SP cells into root canals after pulpectomy. These results demonstrate the utility of TRH-DE as a novel dental pulp biomarker during regeneration of pulp.

**Keywords:** Thyrotropin-releasing hormone degrading enzyme (TRH-DE); Dental pulp tissue; Pulp regeneration; Dental pulp progenitor/stem cells; Biomarker; $CD31^-$ side population (SP) cells; $CD105^+$ cells

**Abbreviations:** RT-PCR: Reverse Transcription-Polymerase Chain Reaction; SP Cells: Side Population Cells; TRH-DE: Thyrotropin-Releasing Hormone Degrading Enzyme

## Introduction

The regeneration of dental pulp after pulpectomy in teeth with complete root formation including induction of apical closure and dentin bridge formation restored the vitality of teeth [1]. In endodontics and conservative dentistry it is beneficial to reduce the risk of periapical disease, root fracture and postoperative pain. A new era in regenerative endodontic therapy has been ushered by the recent success in pulp regeneration after transplantation of pulp $CD105^+$ stem/progenitor cells with SDF-1 after pulpectomy [2]. At present there is no specific biomarker to identify and qualify the regenerated pulp tissue, although nonspecific markers *Syndecan 3* and *Tenascin C* are expressed [3,4]. The newly regenerated pulp tissue has demonstrated novel protein and mRNA expression patterns [2]. A comprehensive and efficient protocol for identification and quantitation of specific biomarkers in regenerated dental pulp remains a major challenge. Approaches for discovery of novel biomarkers include RNA expression, protein expression, metabolomics and functional imaging [5]. A comprehensive approach to biomarker identification consists of the covalent linkage of potentially accessible proteins to biotin molecules followed by their purification, analysis of isolated glycopeptides and nonglycosylated peptides [6]. Successful identification of markers for tissue engineered articular cartilage has been reported by a genome-wide expression analysis of RNA isolated from articular and growth plate (GP) cartilage [7]. The dental pulp tissue is surrounded by periodontal ligament and gingiva, all of which are developmentally originate from the neural crest. The transcript profiling of periodontal ligament fibroblasts were compared with gingival fibroblasts, indicating distinction between the two cell types [8]. During the course of systematic transcript profiling of human dental pulp in comparison to periodontal ligament and gingiva, we identified TRH-DE in dental pulp by microarray analysis and real time RT-PCR analysis. Gene expression profiling of regenerated pulp after pulp stem/progenitor cell transplantation demonstrated that TRH-DE is a novel biomarker for regenerated dental pulp.

## Material and Methods

### Gene expression analyses

The study was approved by the university and institutional ethics committee of Kyushu University, Aichi-gakuin University and National Center for Geriatrics and Gerontology, Research Institute. Human third molars were extracted after obtaining written informed consent from all individuals. Total RNA was extracted from dental pulp tissue, periodontal ligament and gingiva of the same teeth, respectively, using Trizol (Invitrogen). For microarray analysis biotinylated cRNA were prepared from 250ng of total RNA of each tissue according to the standard Affymetrix protocol (Affymetrix Japan K.K., Tokyo, Japan). Following fragmentation, 10μg of cRNA were hybridized for 16 hours at 45°C on GeneChip Human Genome U133Plus 2.0 Array 900470 (Affymetrix) containing ~23,000 annotated sequences.

(http://www.affymetrix.com/jp/products_services/array/specific/hgu133plus.affx#1_4).

GeneChips were washed and stained in the Affymetrix Fluidics Station 450, and were scanned using the Affymetrix GCS3000 scanner. The data were analyzed with Microarray Suite version 5.0 (MAS 5.0) using Affymetrix default analysis settings and global scaling as

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normalization method. The trimmed mean target intensity of each array was set to 500.

The gene expression profile of the dental pulp was compared with those of periodontal ligament and gingiva, and the genes which expressed at higher level in the dental pulp were identified. Expression of those genes in many variety of tissue was examined using the online protein atlas (http://www.proteinatlas.org), and one gene, thyrotropin-releasing hormone (TRH)-degrading enzyme (DE) which has been reported to express in very limited tissue was selected to be further evaluated as a possible pulp tissue marker.

### Real time reverse transcription-polymerase chain reaction analysis

To examine localized expression of human TRH-DE human total RNA (Invitrogen, Corp, Carlsbad, CA) and the total RNA from human pulp tissue were used. First-strand cDNA syntheses were performed from total RNA by reverse transcription using the ReverTra Ace-α (Toyobo, Tokyo, Japan). Real time RT-PCR amplifications were performed at 95°C for 10 sec, 62°C for 15 sec, 72°C for 8 sec using human TRH-DE (forward 5'-CCAACAGGCATCAACACTTA-3', reverse 5'-CCTGTCATCACTGCAAGTTA-3') labeled with Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics, Pleasanton, CA) in Light Cycler (Roche Diagnostics). The RT-PCR product was confirmed by sequencing based on published human cDNA sequences. The expression in variety of human tissue was compared with that in human pulp tissue after normalizing with β-actin (forward 5'-GGACTTCGAGCAAGAGATGG-3', reverse 5'-AGCACTGTGTTGGCGTACAG-3').

Canine dental pulp cells were separated from upper canine teeth. The stem/progenitor cells, $CD31^-$ SP cells and $CD105^+$ cells from pulp cells were isolated at the second passage as previously described [2, 9]. Neuronal induction from pulp $CD31^-$ SP cells was performed at the fifth passage as previously described [9]. First-strand cDNA syntheses were performed from total RNA extracted from those above cells and unfractionated total pulp cells at the fifth passage. The number of these cells was normalized to $5\times10^4$ cells in each experiment. Canine TRH-DE primer set (forward 5'- CCAGCAGGCATCAACACTTA-3', reverse 5'- CCTGTCATCGCTGCAAGTTA-3') was used for real time RT-PCR, based on published cDNA sequence in the GenBank (submitted). Expression of TRH-DE in pulp $CD31^-$ SP cells and $CD105^+$ cells was compared with unfractionated total pulp cells and induced neuronal cells after normalizing with β -actin.

### Immunohistochemical analysis

Normal dental pulp tissues were extracted from human normal third molars, fixed in 4% paraformaldehyde solution (Nakarai Tesq, Kyoto, Japan) overnight, and immersed into 15% sucrose solution and further into 30% sucrose solution and were embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura, Tokyo, Japan). Frozen sections, 12 μm in thickness, were cut and mounted on APS-coated slides (Matsunami, Osaka, Japan). For TRH-DE immunostaining, the endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 minutes. To avoid nonspecific staining, the sections were preincubated in blocking solution (PBS containing 5% normal rabbit serum) for 2 hours at room temperature (RT), and incubated with antibody against TRH-DE (N-18) (1:100, Goat-anti-human, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at RT. After washing three times with PBS, sections were incubated in secondary antibody, a biotinylated rabbit anti-goat IgG (1:200, Invitrogen) for 1 hour at RT. Adjacent sections were used for neuronal staining and H.E. staining. For neuronal staining, the frozen sections were incubated for 15 min with 0.3% Triton X-100 (Sigma Chemical; St Louis, MO), treated with 2.0% normal goat serum to block non-specific binding, and further incubated with rabbit anti-human PGP9.5 (Ultra Clone Ltd.) (1:15,000) at 4°C over night. After washing, bound antibodies were reacted with biotinylated goat anti-rabbit IgG secondary antibody (Vector) (1:200) for 1 hour at room temperature. In the control sections all procedures were processed in the same manner, but the primary antibodies were omitted. The sections were also developed with the ABC reagent using the DAB chromogen for 10 min., counterstained with hematoxylin, and photographed on a microscope (Leica, 6000B-4).

### In situ hybridization and immunohistochemical double staining

To further examine colocalization of TRH-DE expressed cells and neuronal process, double staining of TRH-DE mRNA and PGP9.5 was performed. The human probe for TRH-DE (204 bp) was constructed out of the plasmid after subcloning the PCR products using the primer set (forward 5'-AAGAGCAGCTCACCCTTCG-3', reverse 5'-GCACAGCGAGCATTGTGAC-3'). Human cDNA of TRH-DE, linearized with *Spe* I and *Nco* I were transcribed in vitro in the presence of Digoxigenin for production of anti-sense and sense probes with T7 and SP6 polymerases, respectively. In situ hybridization staining of 12 μm-thick frozen sections of human normal pulp tissue was done as previously described [10]. In brief, the sections were treated with 20 μ/ml proteinase K for 6 min and hybridized with TRH-DE antisense and sense probes in hybridization solution at 57°C for 15 hours. Then, after treatment with RNase buffer at a concentration of 20 μg/ml, the sections were washed in high stringency buffer, 2×SSC and 0.1×SSC, each for 20 min. They were incubated with anti-DIG-HRP (Enzo Biochem, New York, NY) after equilibration in TNB buffer (pH 7.5), and further developed in Rodamine Tyramide working solution (PerkinElmer, Boston, MA) for 10 min. Furthermore, the sections were blocked with 2.0% normal goat serum, incubated with rabbit anti-human PGP9.5 (1:10,000) at 4°C overnight, and reacted with donkey anti-rabbit IgG-FITC secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:100) for 30 min at room temperature. In the control sections all procedures were processed in the same manner, but the primary antibodies were omitted and/or sense probes were used. They were examined by confocal laser microscopy (TCS SP5 conventional inverted microscope, Leica). Three dimensional structures were reconstructed by Leica Application Suite Advanced Fluorescence (LAS AF) software.

### Regulation of TRH-DE mRNA expression in vitro in cell culture by TRH and neuropeptide Y

Canine unfractionated total pulp cells were separated as previously described [9]. The cells at the fifth passage of culture were treated with thyrotropin-releasing-hormone (TRH) ($10^{-7}$ M, Sigma), α Calcitonin Gene Related Peptide (αCGRP) ($10^{-7}$ M, Sigma), and neuropeptide Y ($3\times10^{-6}$ M, Phoenix Pharmaceuticals, Inc.) in Dulbecco's modified Eagle medium (DMEM) with 10 % fetal bovine serum (Invitrogen Corp.). Total RNA was extracted from those cells 24 hours after treatment, and expression of TRH-DE mRNA was determined by real time RT-PCR using the canine TRH-DE primer set. Expression of TRH-DE in canine pulp $CD31^-$ SP cells treated with TRH, αCGRP and neuropeptide Y and PMA was compared with that in those with after normalizing with β-actin.

### Expression of TRH-DE mRNA in the canine regenerated tissue

To further examine whether TRH-DE is a possible biomarker for pulp tissue, expression of TRH-DE in the regenerated tissue after pulp CD31$^-$ SP cell transplantation was compared that in the normal pulp tissue. The autologous cell transplantation into pulpectomized root canals with complete apical closure was performed as previously described [2]. The canine probes for TRH-DE (613bp) was constructed out of the plasmid after subcloning the PCR products using the primer sets (forward 5'- GGCTGGAACATGGAAAAACA-3', reverse 5'-GATTGATGTTCCCCAGCAAC-3'). Canine cDNA of TRH-DE, linearized with *Nco* I and *Spe* I were transcribed in vitro in the presence of Digoxigenin for production of anti-sense and sense probes with SP6 and T7 polymerases, respectively. In situ hybridization analysis was performed in 12 μm-thick frozen sections of the canine regenerated tissue and normal pulp tissue as described above. Real-time RT-PCR was performed in the regenerated tissue on day 28 compared with normal pulp and periodontal ligament using canine TRH-DE primers.

The experimental protocol was approved by the Animal Care and Use Committee of Aichi-gakuin University and National Center for Geriatrics and Gerontology, Research Institute and National Center for Geriatrics and Gerontology, Research Institute.

### Statistical analyses

Data are reported as means ± SD. Statistical significance was analyzed by one-way factorial analysis of variance and multiple comparison test (Tukey's test). The number of replicates in each experiment is indicated in the figure legends.

## Results

### Gene expression profile in human dental pulp

We compared the gene expression patterns of dental pulp tissue with periodontal ligament and gingiva. A 10-fold higher gene expression in dental pulp compared with both periodontal ligament and gingiva was used as the experimental cut-off value that would be significant. There were seven genes that were elevated including deleted in azoospermia (DAZ), sal-like 1 (SALL1), low density lipoprotein-related protein 1B (LRP1B), thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE)/pyroglutamyl peptidase II (PPII), and G protein-coupled receptor 37 (endothelin receptor type B-like) (GPR37), and two unknown genes (Table 1). Expression of these genes in a variety of human tissues was examined using the online protein atlas (http://www.proteinatlas.org). Among those genes, TRH-DE was selected for further evaluation as a potential pulp tissue biomarker. The expression of TRH-DE in various tissues in the human body, real-time RT-PCR analysis was performed. The highest level of TRH-DE mRNA was found in brain, cerebellum and dental pulp compared to other tissues (Figure 1).

### TRH-DE mRNA expression in dental pulp

The expression of TRH-DE mRNA in pulp stem/progenitor CD31$^-$ SP cells and CD105$^+$ cells, was similar to unfractionated total pulp cells. On the other hand, significantly higher level of TRH-DE mRNA was found in induced neurons from pulp CD31$^-$ SP cells, demonstrating it as genuine neuronal marker (Figure 2A).

Localized expression of TRH-DE was found in neuronal process and weakly in pulp cells in intact human pulp tissue by immunohistochemistry (Figure 2B). Similar expression pattern was detected by immunostaining of a neuronal marker, PGP9.5, using the adjacent sections (Figure 2C). Double staining of TRH-DE mRNA by in situ hybridization and PGP9.5 by immunohistochemistry showed that TRH-DE mRNA co-localized with PGP9.5, suggesting TRH-DE expression in nerve fibers in dental pulp (Figures 2D-G).

### Regulation of TRH-DE mRNA in pulp cells by TRH, CGRP and Neuropeptide Y

A significant decrease in TRH-DE mRNA was observed in unfractionated total pulp cells following treatment with $10^{-7}$ M TRH in cell culture. However, αCGRP, which is expressed in sensory nerve fiber, did not increase the level of TRH-DE mRNA. It is noteworthy that Neuropeptide Y, which is expressed in sympathetic nerve fiber, significantly increased TRH-DE mRNA in pulp cells (Figure 3).

### Expression of TRH-DE in regenerated pulp tissue compared with normal pulp tissue

We have previously reported that pulp-like tissue was regenerated after transplantation of pulp CD105$^+$ stem/progenitor cells into pulpectomized root canals in dogs [2]. In the present investigation, pulp CD31$^-$ SP cell transplantation after pulpectomy into the root canals formed new pulp-like tissue with nerves and vasculature by day 28 (Figure 4A, B). Staining with BS-1 lectin demonstrated neovascularization in the regenerated tissue by confocal laser microscopy (Figure 4C). The regenerated tissue contained spindle shaped cells in the upper part

| Name | Description | Gene Bank | Relative Expression | |
|---|---|---|---|---|
| | | | pulp / gingiva | pulp / periodontal ligament |
| DAZ | Deleted in azoospermia | NM_004081.2 | 70.97 | 50.20 |
| SALL1 | sal (Drosophila)-like 1 | NM_002968.1 | 10.02 | 10.11 |
| LRP1B | low density lipoprotein-related protein 1B (deleted in tumors) | NM_018557.1 | 116.36 | 15.66 |
| TRH-DE | thyrotropin-releasing hormone degrading ectoenzyme | NM_013381.1 | 776.54 | 28.13 |
| GPR37 | G protein-coupled receptor 37 (endothelin receptor type B-like) | U87460.1 | 81.80 | 12.48 |
| Unknown | - | AW082827 | 18.11 | 15.39 |
| Unknown | - | BE466525 | 10.31 | 38.33 |

**Table1:** Highly expressed genes in dental pulp compared with gingiva and periodontal ligament by microarray analyses.

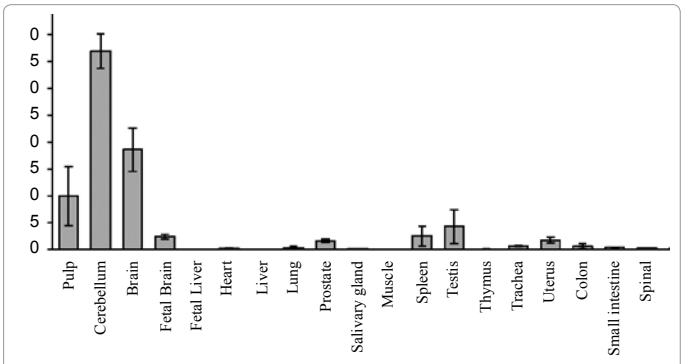


**Figure 1: Relative mRNA expression of *thyrotropin-releasing hormone (TRH)-degrading enzyme (DE)* by real-time reverse transcription-polymerase chain reaction in various human tissues.** The highest level of *TRH-DE* expression in cerebellum, brain and dental pulp compared with other tissues. The experiment was repeated three times. Data are expressed as means ± SD derived from three independent experiments.

and stellate-like cells in the middle part similar to those in the normal pulp. The odontoblast-like cells were found to attach to the dentinal wall in the root canal, extending their processes into dentin tubules (Figure 4D). The neuronal process stained by PGP9.5 antibody was extended into the newly regenerated pulp from apical foramen (Figure 4E). Thus, these results demonstrate the morphological characteristics of dentin-pulp complex in the regenerated pulp.

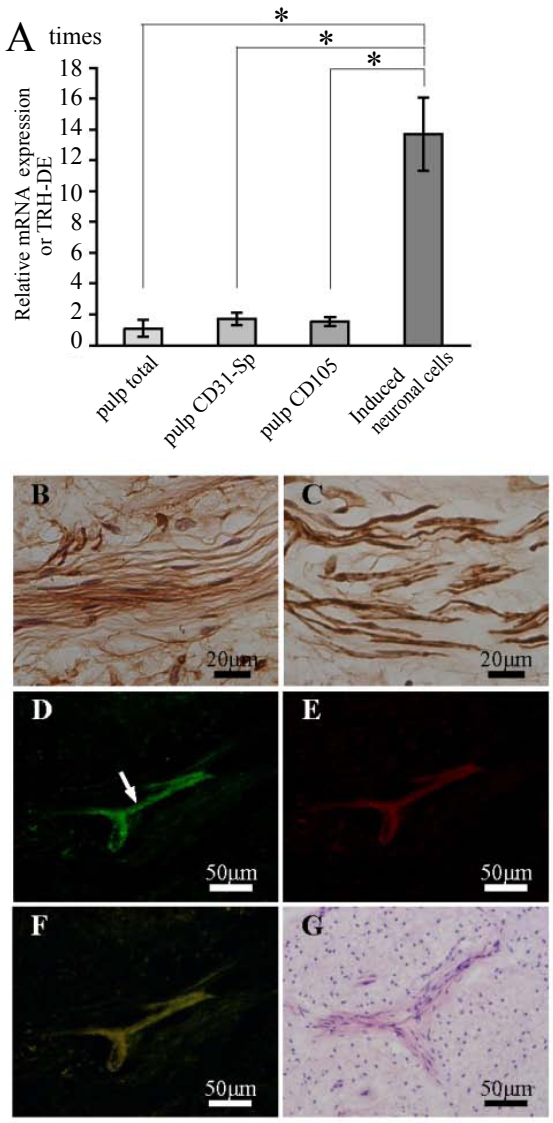


**Figure 2: mRNA expression of *TRH-DE* in human dental pulp**

A: Higher expression of *TRH-DE* mRNA in induced neuronal cells from canine pulp CD31$^-$ SP cells compared to unfractionated total pulp cells, pulp stem/progenitor CD31$^-$ side population (SP) cells, CD105$^+$ cells. Data are expressed as means ± SD at 4 determinations (*$P$<0.005).

B, C: Immunohistochemical analysis of localization of TRH-DE (B) and PGP9.5 (C) in human normal pulp tisssue. TRH-DE protein is expressed in neuronal processes.

D-F: Immunohistochemical and in situ hybridization analyses in human normal pulp tisssue by confocal microscopy. PGP9.5 (D), *TRH-DE* mRNA (E) and Merge (F). *TRH-DE* mRNA expression overlapped PGP 9.5 in neuronal process.

G: H&E staining in the adjacent section to (D-F). Arrows indicate neuronal processes.

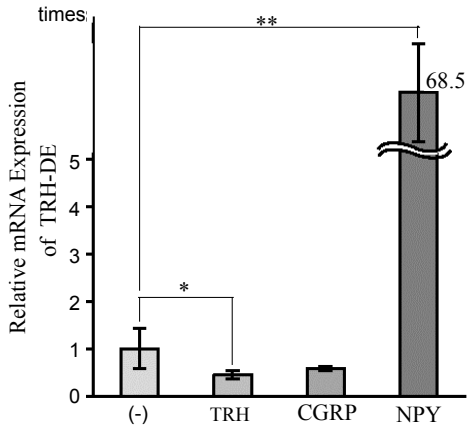


**Figure 3: Regulation of *TRH-DE* mRNA in human pulp cells.** Enhanced expression of *TRH-DE* mRNA 24 hours after treatment with $3\times10^{-6}$ M neuropeptide Y (NPY), and significantly lower expression with $10^{-7}$ M thyrotropin-releasing hormone (TRH) in canine unfractionated total pulp cells.Little effect by $10^{-7}$ M α Calcitonin Gene Related Peptide (CGRP). Data are expressed as means ± SD derived from three independent experiments (*$P$<0.05, **$P$<0.001). The experiment was repeated three times, and one representative experiment is presented.

TRH-DE and PGP9.5 were localized by immunohistochemistry in the adjacent sections on nerve fibers in the regenerated pulp as in normal pulp (Figures 4F, G). In addition, there was colocalization of TRH-DE mRNA expression with PGP9.5 in the regenerated pulp by in situ hybridization and immunohistochemistry (Figure 4H), as in normal canine pulp (Figure 4I). The localization of TRH-DE mRNA was identical in both normal and regenerated dental pulp. However, in tissues from the same human subjects TRH-DE was significantly higher in pulp compared to periodontal ligament (Figure 4J). In conclusion, the results demonstrate that TRH-DE is a bonafide biomarker in both normal and regenerated pulp.

## Discussion

TRH-DE, also known as pyroglutamyl peptidase II, is a member of M1 family of Zn-dependent aminopeptidases, and displays absolute functional specificity for its substrate, TRH [11,12]. TRH is an important multifunctional signaling molecule in the CNS [13], regulating energy homeostasis, feeding behavior, thermogenesis, locomotor activation and autonomic regulation. These CNS-mediated actions are independent of the hypothalamic-pituitary-thyroid (HPT) axis [14,15]. TRH-DE is an extracellularly oriented, membrane-associated peptidase (ectopeptidase) and located to terminate peptide-mediated cell signaling [16,17]. TRH-DE is widely distributed in brain, specifically in neuronal cells, and significant activities have been also detected in adenohypophysis (pituitary), retina, lung and liver, but not in other tissues, such as heart, kidney and muscle [18-23]. Northern blot analysis demonstrates that tissue distribution of mRNA levels paralleled the distribution of enzymatic activity [24]. Thus, this investigation has demonstrated for the first time that TRH-DE mRNA is strongly expressed in dental pulp as in brain.

TRH is hydrolyzed by the membrane-bound TRH-DE in monolayer cultures from brain and reaggregate cell cultures of anterior pituitary [11]. Relatively high enzymatic activity is associated with cultured neuronal cells from embryonic brain while glial cells are almost devoid of the activity. A significant activity is found on anterior pituitary cell aggregates. TRH-DE on adenohypophyseal cell is regulated by estradiol and tightly regulated by triiodothyronine (T3). The tissue-

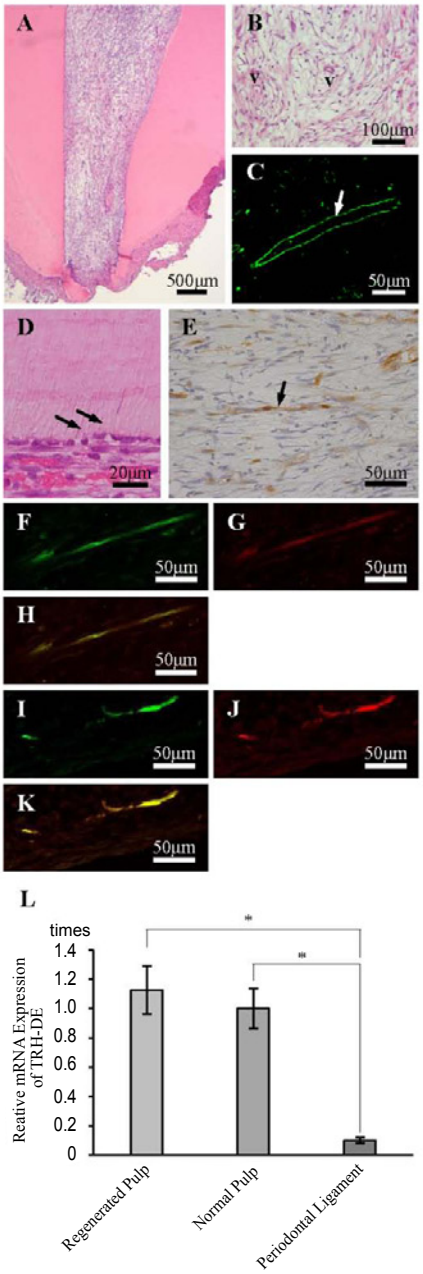


**Figure 4: Expression of TRH-DE in canine regenerated pulp tissue compared with normal pulp tissue**

A, B: Complete pulp regeneration 28 days after autologous transplantation of CD31- SP cells with SDF-1. H&E staining.Low power (A). High power regenerated pulp showing stellate-like pulp cells and vessel (v) (B).

C: Immunostaining with BS-1 lectin. Arrow indicates vessel.

D: Odontoblast-like cells extending their process (arrows) into dentinal tubules, showing characteristics of dentin-pulp complex. H&E staining.

E: PGP9.5 immunohistochemistry. Arrow indicates nerve fibers.

F-K: Confocal microscopic analysis of colocalization of PGP9.5 (F, I) with *TRH-DE* mRNA(G, J) by immunohistochemistry and in situ hybridization. Merge image (H, K). *TRH-DE* mRNA expression overlapped PGP9.5 positive nerve fibers in regenerated pulp tissue (I-K) as normal pulp tissue (F-H).

L: Similar mRNA expression of *TRH-DE* in regenerated pulp tissue to that in normal pulp, and significant difference from that in periodontal ligament. Data are expressed as means ± SD at 4 determinations (*$P$<0.01).

specific regulation and very heterogenous distribution of TRH-DE in brain and pituitary cells is suggestive that TRH-DE may function in the transmission of TRH signals at specific target sites [11]. TRH specifically downregulates TRH-DE activity in adenohypophyseal cells through TRH receptor activation, and the TRH effect is mimicked by calcium-independent protein kinase C (PKC) activator, phorbol-12-myristate-13-acetate (PMA) [25]. After neuronal degeneration of serotonin-TRHergic cells that project from raphe nuclei to the spinal cord, TRH-levels is decreased, but no change of TRH-DE activity, suggesting localization of TRH-DE not in TRHergic neuron but in the target cells [26]. It is generally agreed that TRH not only functions as a hypothalamic hypophysiotrophic hormone but also as a neurotransmitter/modulator in extrahypothalamic areas [27]. TRH has neurotropic effects [28] and an effective mechanism must exist for termination of its action. On the basis of its high substrate specificity, active site orientation, synaptosomal location TRH-DE provides a specific and efficient mechanism for inactivation of neuronally released TRH. The presence of neuropeptide Y in human pulp from sympathetic nerves originating in the superior cervical ganglion has been reported [29,30]. Neuropeptide Y is co-stored with norepinephrine in sympathetic neurons, and sympathetic nerve activation, such as stress, physical exercise, life-threatening situations, stimulate its release together [31,32]. Dental caries and thermal/mechanical irritants of dentin-pulp complex also stimulate neuropeptide Y release [33]. Neuropeptide Y is thought to play an important role in regulating the pulp inflammation by modulating circulation [34]. Its receptor is localized to the blood vessel and the nerve fiber [34]. In the present study, we demonstrated the localization of TRH-DE to the nerve fibers in the dental pulp and the up-regulatory effect of neuropeptide Y on TRH-DE. Taken together these facts, TRH-DE may be involved in inflammatory actions of neuropeptide Y in the pulp.

The biological actions of neuropeptides are terminated or transformed by proteolytic enzymes, and the bioavailability of neuronally released peptides is predominantly controlled by peptide-degrading ectoenzymes located on synaptosomal membranes [35]. TRH down-regulates TRH-DE mRNA levels in GH3 rat pituitary tumor cells and primary adenohypophyseal cells [36]. In the present study TRH-DE markedly decreased the level of TRH-DE mRNA in total pulp cells, revealing fundamental similarities in the mechanism of TRH-DE mRNA to pituitary cells. The down-regulatory effect of TRH and the up-regulatory effect of neuropeptide Y on its degrading enzyme, TRH-DE may provide a positive feedback mechanism to insure the availability and lasting actions of neuropeptides, TRH and neuropeptide Y on target cells in the dental pulp.

Dental pulp has many important functions in the homeostasis and maintenance of teeth. Vasculature in the pulp tissue plays a critical role in nutrition and oxygen supply, function as a conduit for the transport of metabolic waste and regulating inflammation. Nerve fibers in the pulp tissue contribute to angiogenesis, extravasation of immune cells to regulate inflammation, pulp homeostatis, and pulp defense mechanisms. Innervation and vasculature of the dental pulp were intimately associated in pulp homeostatis. Thus, angiogenesis/vasculogenesis and neurogenesis are indispensable for pulp regeneration [36]. In the present investigation TRH-DE was identified as a biomarker in nerves in the normal and regenerated pulp tissue. Enzymatic assays for TRH-DE [37] might be a critical advance over RNA and proteomic analysis. In conclusion, the identification of TRH-DE as a diagnostic biomarker of regenerating dental pulp represents an important advance in dental regenerative medicine and tissue engineering.

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