RESEARCH ARTICLE

Expression of $GABA_A$ Receptor Subunits α1 and β2 in Healthy Human Dental Pulp

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Abstract

BACKGROUND: Gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the mammalian central nervous system (CNS), has a well-established role in pain modulation. While numerous studies have delved into the expression of GABA and its receptors in dental pulp, the exact influence of these receptors on dental pain signaling has not been fully elucidated. This study aimed to investigate the gene and protein expression of the two most abundantly expressed GABA type A ($GABA_A$) receptor subunits, $GABA_A$ receptor subunit α1 (GABRA1) and β2 (GABRB2), in healthy human dental pulp.

METHODS: Six tooth samples were collected from healthy individuals referred for orthodontic treatment. Total RNA was isolated from the pulp tissues of three samples and reverse transcription-polymerase chain reaction (RT-PCR) was performed to assess gene expression of *GABRA1* and *GABRB2*. The other three samples were examined using immunohistochemistry (IHC) for visualization of GABRA1 and GABRB2 proteins within the dental pulp.

RESULTS: RT-PCR analysis reported the presence of both *GABRA1* and *GABRB2* in the dental pulp, and independent t-test analysis revealed that the expression of *GABRA1* was significantly higher than *GABRB2*. The immunohistochemical staining provided compelling visual evidence of the expression of GABRA1 and GABRB2 proteins within the odontoblast layer of dental pulp, clearly indicating their presence in the cell bodies and odontoblastic processes extending into the dentin.

CONCLUSION: The presence of α1 and β2 subunits of the $GABA_A$ receptor in healthy human dental pulp offers valuable insights for further research into the potential roles of $GABA_A$ receptors in dental pain signaling.

KEYWORDS: γ-aminobutyric acid, $GABA_A$ receptors, GABRA1, GABRB2, dental pain, pain signaling

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Introduction

The dental pulp is the innermost layer of the tooth tissue that contains various structures, including nervous tissue, blood vessels, connective tissues and various types of specialized cells. The pulp plays a crucial role in maintaining tooth vitality and its primary functions can be broadly classified into three main categories. The first one is defence process: dental pulp can initiate an inflammatory response upon detection of bacteria or foreign bodies within the tooth. This response can help fight infection and protect the tooth from further damage.(1) The second one is dentine formation: the dental pulp contributes to the continuous deposition of dentin throughout the life of the tooth, thus helping maintain the structural integrity of the tooth.(2) The third one is the sensory functions: the dental pulp contains nerves that transmit sensory signals to the brain. These sensory nerves allow the tooth to detect and respond to external stimuli, including changes in temperature, pressure and pain.

(3) This highly vascularized and innervated soft tissue is protected by the hard and mineralized enamel and dentin layers.(4) However, when the protective enamel and dentin layers are compromised due to factors like caries, trauma, inflammation, or periodontal diseases, the dental pulp can become exposed to external stimuli, leading to severe pain and various dental problems.(5)

Although extensive efforts have been made to understand the cellular and molecular mechanisms underlying dental pain, these mechanisms are not fully known, thus making it challenging to devise effective dental pain management strategies. However, recent studies have shed light on the involvement of the transient receptor potential (TRP) channels in dental pain signaling.(6) These non-selective ion channels found in dental neurons and odontoblasts are believed to play a role in transmitting various stimuli related to dental pain. Preclinical and clinical studies targeting TRP channels, particularly TRPV1 receptors, have demonstrated the potential analgesic effects of TRPV1 receptor agonists.(7,8) Given the progress in researching TRP channels, similar approaches may be applicable to other ion channels in dental pulp for the development of targeted pain relief medications.

Gamma-aminobutyric acid (GABA) type A ($GABA_A$) receptors are pentameric ligand-gated Cl^- ion channels that are activated by the inhibitory neurotransmitter GABA and play a crucial role in mediating fast synaptic transmissions in the central nervous system (CNS). As $GABA_A$ receptors in mammals can be assembled from 19 different subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3), specific combination of subunits can determine the functional properties, variation in GABA sensitivity and pharmacological profile of a $GABA_A$ receptor. Therefore, identifying the specific subunit composition of $GABA_A$ receptors in the dental pulp is integral for designing drugs that selectively target certain receptor isoforms while avoiding or minimizing interactions with other subunits that may be associated with undesired outcomes.(9)

Several studies have established that functional $GABA_A$ contain at least one α, one β and one additional subunit.(10) This due to the fact that the GABA binding site is present at the interface between α and β subunits. Although the majority of $GABA_A$ receptors consist of two α subunits, two β subunits, and one γ subunit, variations in subunit subtypes yield different configurations of the 2α:2β:1γ conformation. (11) The α2β2γ1 subtype is predominant in the mammalian CNS, constituting 60% of all $GABA_A$ receptors and being present both synaptically and extra-synaptically.(11) Of the various α and β subunits that make up the $GABA_A$ receptor, $GABA_A$ receptor subunit α1 (GABRA1) and GABAA receptor subunit β2 (GABRB2) have been reported to be the most abundant subunits.(9) While previous studies have reported the presence of GABAA receptor subunit β1 (GABRB1) in the dental pulp, the presence of GABRA1 and GABRB2 in the dental pulp have yet to be reported. (12,13) However, decreased GABRA1 expressions have been observed in other tissues in the body such as in the cartilage tissues of osteoarthritis patients.(14) Similarly, a genome-wide association study has shown that GABRB2 expression has also been associated with hip pain and multisite chronic pain.(15) Considering the role that these subunits play in pain signaling in other tissues, it raises the intriguing possibility that GABRA1 and GABRB2 may play a role in pain signaling within the dental pulp as well. Therefore, this study aimed to investigate the presence of GABRA1 and GABRB2 in healthy human dental pulp.

Methods

Tooth Collection and Sample Preparation

A total of six sound molars extracted for orthodontic reasons were collected from 13- to 25-year-old patients treated at Hospital Universiti Sains Malaysia (USM), Malaysia. This study was approved by Jawatankuasa Etika Penyelidikan Manusia (JEPeM), USM (No. USM/JEPEM/20090458) in 2020 and was performed in accordance with the guidelines outlined by the committee. From a total of six tooth samples that were collected, three samples were used for reverse transcription-polymerase chain reaction (RT-PCR) analysis, and the other three were used for immunohistochemistry (IHC). For the RT-PCR analysis, fresh tooth samples were split longitudinally using a hard tissue cutter and the dental pulp was gently removed using sharp tweezers. The dental pulp was then placed in phosphate-buffered saline before proceeding with RNA isolation. For IHC, the collected samples were fixed in 4% paraformaldehyde for 24 hours at 4°C before being placed in 5% nitric acid until complete decalcification was achieved. Following the decalcification process, samples were processed, embedded in paraffin and kept at 4°C until they were ready to be sectioned.

RNA Isolation and RT-PCR

Total RNA was extracted from dental pulp samples using the RNeasy® Fibrous Mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions and RT-PCR was carried out using the QIAGEN® OneStep RT-PCR kit (Qiagen). Briefly, each PCR reaction contained 60 ng of RNA

template, 5 µL of 5× QIAGEN OneStep RT-PCR Buffer, 1 µL dNTP Mix, 1 µL of both 15 µM forward and reverse primers and 1 µL QIAGEN OneStep RT-PCR Enzyme Mix. The reaction mixture was then made up to a total volume of 20 µL with RNA-free water. β-actin (*ACTB*) was used as the positive control and the template was omitted for the negative control. The primers, annealing temperatures and expected PCR product size were detailed in Table 1. The PCR products were then loaded onto 2% agarose gels stained with SYBR™ Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and run at 70V for 1 hour. Gels were then visualized and imaged using the FluorChem M (Bio-Techne, Minneapolis, MN, USA) imaging system. The signal intensity of each band was then measured using ImageJ Version 1.53t (NIH, Bethesda, MD, USA) and the intensity of each band relative to the control *ACTB* band was calculated.

IHC

Sections of 2.5 µm thickness were cut from the paraffin blocks and mounted on polylysine-coated glass slides. The sections were then dewaxed in xylene, rehydrated in a descending series of ethanol and subjected to heat-mediated antigen retrieval with tris-ETDA buffer (pH 9.0) in a decloaking chamber. Following antigen retrieval, sections were treated with 3% hydrogen peroxide solution for 10 minutes and subsequently blocked using normal goat serum for 30 minutes. The sections were incubated at 4°C overnight with 1:50 rabbit anti-GABRA1 (Catalog No. E-AB-16446, Elabscience Houston, TX, USA,) and 1:200 rabbit anti-GABRB2 (Catalog No. bs-12065R, Bioss, Woburn, MA, USA) diluted in 5% bovine serum albumin. The next day, primary antibodies were washed off and slides were incubated with polyperoxidase-anti-mouse/rabbit IgG (Elabscience) for 20 minutes. Color development was performed with liquid diaminobenzidine (DAB) chromogen (Elabscience) for 3 minutes and sections were then counterstained with hematoxylin. The stained sections were then dehydrated in increasing concentrations of ethanol and cleared in xylene before being cover slipped. The slides were then visualized and imaged at 200× and 400× magnifications.

Statistical Analysis

SPSS software Version 27 (IBM, Armonk, NY, USA) was used to perform statistical analysis. Shapiro-Wilk test was carried out to determine the normality and the data was found to be normally distributed. Independent t-test was then carried out and the results were expressed as the mean and standard deviation (SD). All statistical analyses were performed at the significance level of $p<0.05$.

Results

GABRA1 and *GABRB2* was Expressed in the Human Dental Pulp

RT-PCR analysis indicated that *GABRA1* and *GABRB2* were present in all three human dental pulp samples that were studied. Figure 1 showed the agarose gel electrophoresis images showing the presence *GABRA1* and *GABRB2* as 66 bp and 408 bp bands that appeared on lane 3 and 4, respectively. *ACTB*, present as a 144 bp band on lane 2, was used as the housekeeping gene and was present in all samples tested. Data obtained from ImageJ (Table 2) was analysed using SPSS. The data was found to be normally distributed and independent t-test results indicated that the expression of *GABRA1* was significantly higher than *GABRB2* (Table 3).

GABRA1 and GABRB2 was Expressed in the Odontoblast Layer of the Human Dental Pulp

Immunohistochemical staining was carried out to determine the expression pattern of GABRA1 and GABRB2 in the human dental pulp. The expression of both proteins were observed in the odontoblast layer throughout the dental

Table 1. Primer sequences used for RT-PCR.

Gene	Primer Sequences (5'-3')	Amplicon Size (bp)	Annealing Temperature (°C)	References
ACTB	Fwd: CCTGGCACCCAGCACAAT Rev: GGGCCGGACTCGTCATAC	144	60	(16)
GABRA1	Fwd: GGATTGGGAGAGCGTGTAACC Rev: TGAAACGGGTCCGAAACTG	66	55	(17)
GABRB2	Fwd: GAGTTTTACTGGCGTGGCGATG Rev: GGCATATTCCAGAAGGGCCATG	408	55	(18)

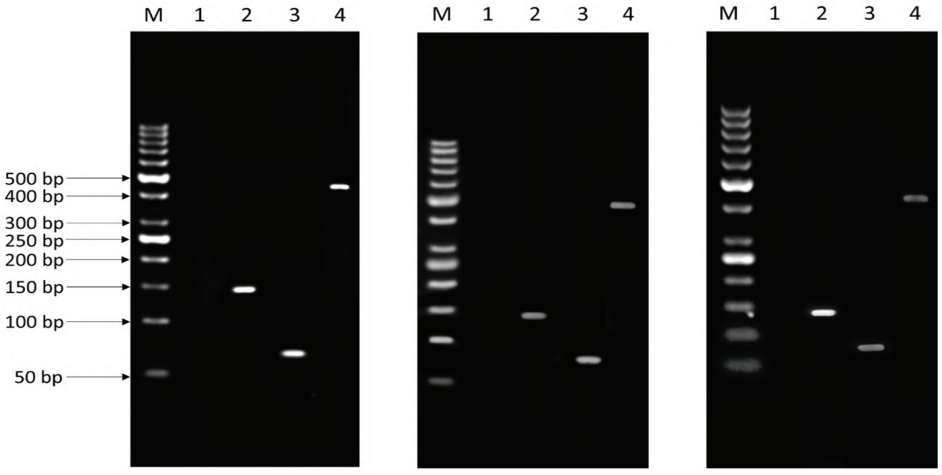

Figure 1. The agarose gel electrophoresis images of biological replicates showing the presence of *ACTB*, *GABRA1* and *GABRB2*. Lane M: 50 bp DNA ladder; Lane 1: Negative control; Lane 2: *ACTB* (144 bp); Lane 3: *GABRA1* (66 bp); Lane 4: *GABRB2* (408 bp).

pulp, as shown in Figure 2 (C,Ci) and Figure 3 (C,Ci), respectively. Staining was mainly localized in the cell bodies of odontoblasts as well as in the odontoblastic processes extending into the dentinal tubules of dentin. In this study, rat spinal cords which have been reported to highly express GABRA1 and GABRB2 in the motor neurons were used as the positive control.(19) As expected, the positive controls showed positive staining in the cell bodies of the motor neuron (Figure 2A,Ai, Figure 3A,Ai) while the negative controls, in which the primary antibodies were omitted, showed no staining (Figure 2B,Bi, Figure 3 B,Bi). These results were consistent across all three tooth samples that were stained. Comparing the staining intensity of both proteins indicated that there was a higher expression of GABRA1 in healthy human dental pulp.

Discussion

The present study aimed to investigate the gene and protein expression of GABRA1 and GABRB2 in the human dental pulp. RT-PCR analysis showed that *GABRA1* and *GABRB2* were indeed present in healthy human dental pulp and relative quantification revealed that *GABRA1* had a significantly higher level of gene expression as compared with *GABRB2*. While it has been reported that the GABRA1 and GABRB2 are the most abundant and are ubiquitously distributed throughout the CNS, it is worth noting that expression levels can vary depending on the specific region or disease state of the particular tissue.(20) This observation aligns with similar findings reported in previous studies, where *GABRA1* was found to exhibit a higher gene expression compared with *GABRB2* in the stomach, bladder and kidney of mice.(21) Such variations in subunit expression has also been observed between brain tissue and peripheral tissues in the context of other receptors. (22) This tissue-specific subunit expression supports the idea that specific subunits may have a more significant role or specific functional implications in different tissues. In two separate studies on healthy human tooth samples, microarray analysis results reported high expression of *GABRB1* in the dental pulp.(12,13) Based on these studies, the lower expression of *GABRB2* compared with *GABRA1*, as reported in the present study, suggests the possibility

Table 2. Area, signal intensity and relative intensity of each band measured using ImageJ.

	Gene	Area (mm^2)	Signal Intensity	Relative Intensity
Gel 1	*ACTB*	156	464015	1.000
	GABRA1	156	426967	0.920
	GABRB2	156	289978	0.625
Gel 2	*ACTB*	156	394552	1.000
	GABRA1	156	361422	0.916
	GABRB2	156	258532	0.655
Gel 3	*ACTB*	156	371920	1.000
	GABRA1	156	317075	0.853
	GABRB2	156	172821	0.465

Table 3. Comparison of *GABRA1* and *GABRB2* gene expression.

Variables	Mean±SD		t-statistic (d.f.)	*p* -value
	GABRA1	*GABRB2*		
Relative Intensity	0.8963±0.0376)	0.5817±0.1021	5.008 (4)	0.007*

of other β subunits being present in the dental pulp which may come together with the α1 subunits to form functional $GABA_A$ receptors.

Immunohistochemical analysis was then carried out to determine protein expression and localization. IHC results revealed that both GABRA1 and GABRB2 were present in the human dental pulp. The proteins were found to be localized at the odontoblast and predentin regions with strong staining seen in the cell bodies as well as the odontoblastic processes in the dentinal tubules. Similar to the gene expression study, a higher expression of GABRA1 was detected. The localization of the proteins observed are consistent with those previous studies which have similarly used IHC to report the existence of a different $GABA_A$ receptor subunit, GABRB1, in the dental pulp.(12,13) Both studies reported that the β1 subunit was also found to be expressed in the odontoblast and predentin layers. This further supports the idea that $GABA_A$ receptors are in fact present in the human dental pulp and are mainly localized in the odontoblast and predentin regions.

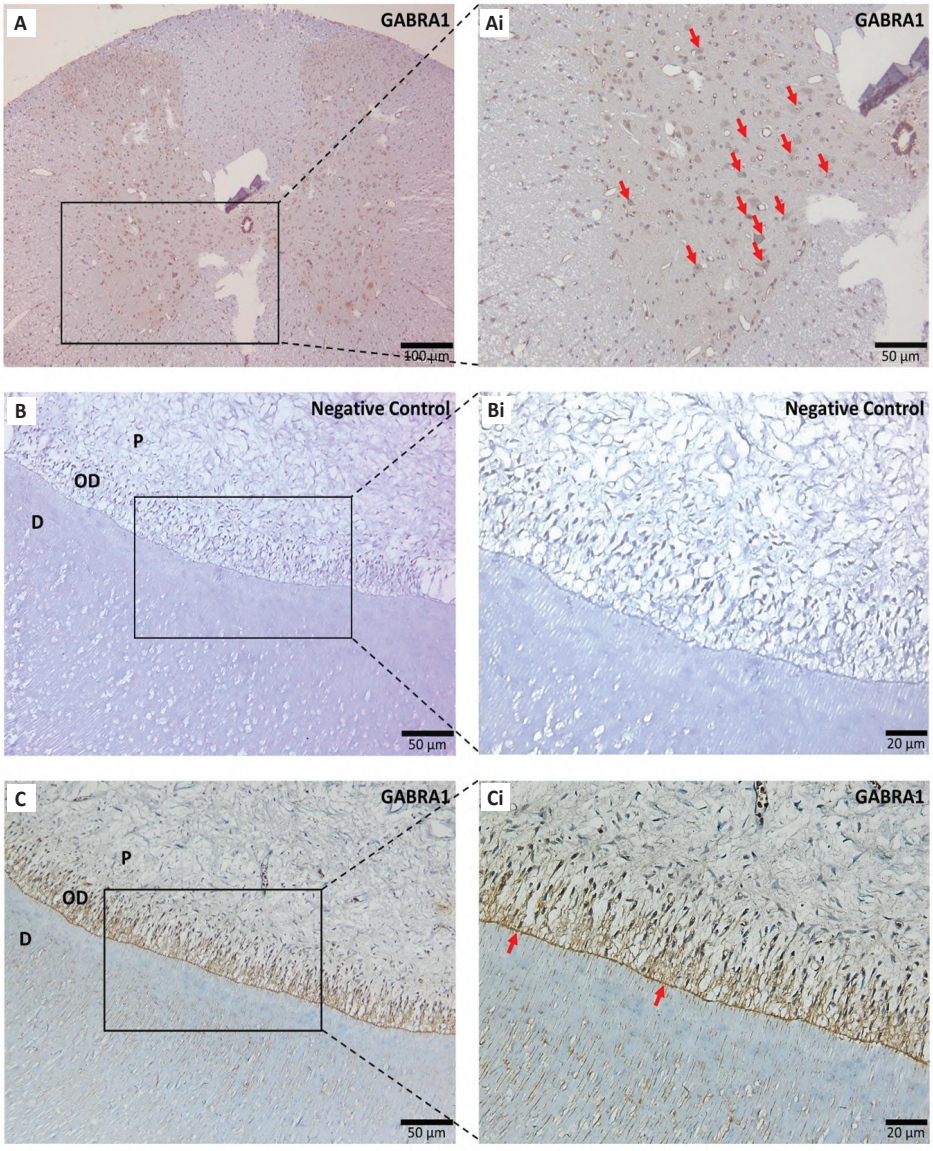

Figure 2. Immunohistochemical localization of GABRA1. (A,Ai) Positive control: GABRA1 was expressed in the cell bodies of rat spinal cord motor neurons (red arrows), (B,Bi) Negative control: No staining was observed, (C,Ci) Tooth sample: GABRA1 was expressed in the odontoblast cells of the tooth samples (red arrows). Brown hue as indicated by the red arrows denotes positive immunostaining for GABRA1 in the respective tissues. All tooth samples examined in this study had similar characteristics. D: Dentin; OD: Odontoblast layer; P: Pulp.

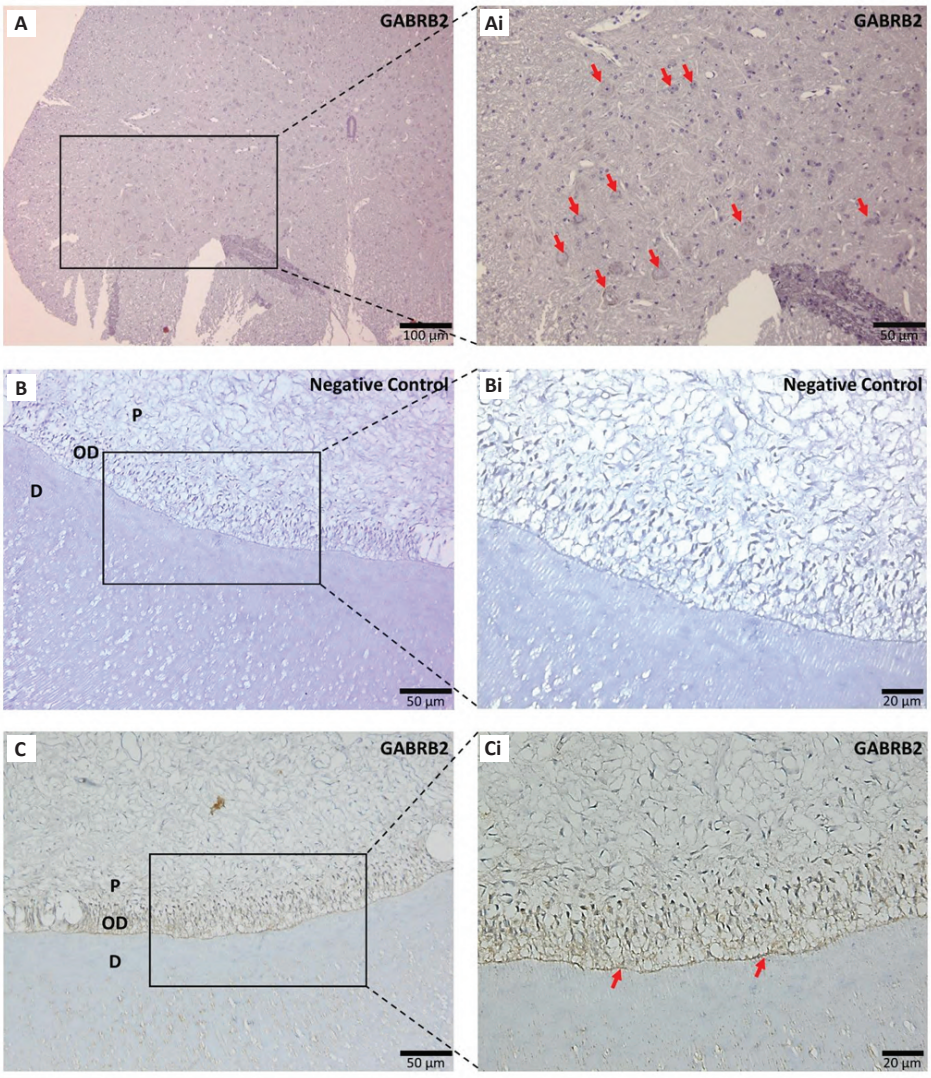

Figure 3. Immunohistochemical localization of GABRB2. (A,Ai) Positive control: GABRB2 was expressed in the cell bodies of rat spinal cord motor neurons (red arrows), (B,Bi) Negative control: No staining was observed, (C,Ci) Tooth sample: GABRB2 was expressed in the odontoblast cells of the tooth samples (red arrows). Brown hue as indicated by the red arrows denotes positive immunostaining for GABRB2 in the respective tissues. All tooth samples examined in this study had similar characteristics. D: Dentin; OD: Odontoblast layer, P; Pulp.

The presence of $GABA_A$ receptors in the peripheral nervous system, along with other GABAergic machineries such as GABA neurotransmitter and the GABA-synthesizing enzymes, glutamic acid decarboxylase 1 (GAD1) and 2 (GAD2), have been found to inhibit pain signaling.(23,24) This is mainly accomplished by the GABAergic signaling filtering the incoming signals from the periphery at the dorsal root ganglion of the principal afferent fibres. This prevents the signals from reaching the spinal cord and being transmitted to the higher pain centers to be perceived.

Based on the results obtained in this study, the presence of GABA receptors in the dental pulp points to the likelihood of a similar GABAergic machinery in the human dental pulp. There have been three main theories that have been proposed to explain the mechanisms underlying dental pain: the hydrodynamic theory, the neural theory and the odontoblast transducer theory.(25) The hydrodynamic theory suggests that external stimuli, like temperature or pressure changes, induces fluid movement within the dentinal tubules that contain odontoblast processes, thus stimulating nerve fibres and causing sensations of pain or discomfort. However, the neural theory suggests that nerve endings found on odontoblast processes within the pulp tissue are directly stimulated by external stimuli. On the other hand, the odontoblast transducer theory proposes that odontoblasts themselves act as receptors for stimuli and transmit signals to the pulp. In all these theories, the odontoblast is the first line of cells that are involved in the pain signaling and the localization of $GABA_A$ receptors in odontoblast cell bodies and processes provides strong support for the hypothesis that GABAergic signaling plays a significant role in dental pain signaling. This also suggests that activation of $GABA_A$ receptors in odontoblasts via $GABA_A$ agonists may lead to the inhibition of pain transmission by reducing the activity of nerve fibres in the pulp.

However, more research is needed to investigate the existence of additional components involved in GABAergic signaling and their possible function in dental pain

signaling. Performing more quantitative gene and protein expression studies such as quantitative PCR or enzyme-linked immunosorbent assay (ELISA) experiments can provide a more precise understanding of $GABA_A$ receptor subunits expression levels in the dental pulp. Colocalization studies of the $GABA_A$ receptor subunits in relation to nociceptive markers, such as neurofilament 200 (NF200), can also be done to provide stronger evidence that $GABA_A$ receptors do play a role in dental pain signaling. Further studies comparing the expression of different $GABA_A$ receptor subunits in diseased and healthy samples can offer insights into the variations in subunit expression associated with specific dental diseases, enabling the modification of pain management strategies to utilize drugs or therapies that might target a particular subunit. Animal studies on dental pain models using $GABA_A$ receptor modulators that target specific subunits, along with pain assessments and neurophysiological recordings, can also facilitate the effective development of dental pain management strategies.

Conclusion

In conclusion, this study has reported the presence of α1 and β2 subunits of the $GABA_A$ receptor in healthy human dental pulp. These results support the idea that GABAergic signaling is involved in dental pain modulation. Further studies looking into the various other elements involved in GABAergic signaling are required in order to develop effective dental pain management strategies targeting the GABAergic system.

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Authors Contribution

DS performed data acquisition and analysis, result interpretation, manuscript preparation and designed the tables and figures. WNSS, NG, TL and RR were involved in interpretation of results as well as the critical revision of the manuscript. RR was involved in concepting and planning the research, manuscript preparation and funding acquisition.

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