EXPRESSION OF INHIBITORY NEUROTRANSMITTER $GABA_A$ RECEPTORS IN HUMAN DENTAL PULP: A POTENTIAL ROLE IN DENTAL NOCICEPTIVE SIGNALLING

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by

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LIST OF SYMBOLS

A	Absorbance
α	Alpha
β	Beta
°C	Degree Celsius
γ	Gamma

LIST OF ABBREVIATIONS

AP	Alkaline phosphatase
ASIC	Acid-sensitive cation channels
BSA	Bovine serum albumin
cDNA	Complementary DNA
CN V	Fifth cranial nerve
CNS	Central nervous system
DAB	Diaminobenzidine tetrahydrochloride
DH	Dentine hypersensitivity
DMP-1	Dentin matrix protein 1
DRG	Dorsal root ganglia
DSPP	Dentin sialophosphoprotein
EDTA	Ethylenediaminetetraacetic acid
FFPE	Formalin fixed paraffin embedded
GABA	Gamma-aminobutyric acid
$GABA_A$	Gamma-aminobutyric acid type A
$GABA_B$	Gamma-aminobutyric acid type B
GABA-T	GABA transaminase
GABRA1	Gamma-aminobutyric acid type A receptor subunit alpha 1
GABRB2	Gamma-aminobutyric acid type A receptor subunit beta 2
GAD	Glutamate decarboxylase
GATs	GABA transporters
GIRK	G protein-gated inwardly rectifying K^+
GRCPs	G-protein-coupled receptors
HCl	Hydrochloric acid
HCN	Hyperpolarisation-activated cyclic nucleotide-gated

HIER	Heat-induced epitope retrieval
HNO3	Nitric acid
HRP	Horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia
ICOP	International Classification of Orofacial Pain
IHC	Immunohistochemistry
IPSP	Inhibitory postsynaptic potential
KCC2	K^+-Cl^- co-transporter 2
KPP-G	Klinik Pakar Pergigian
KRK	Klinik Rawatan Keluarga
NAM	Negative allosteric modulators
NaOH	Sodium hydroxide
NBF	Neutral buffered formalin
NKCCI	Na^+-K^+-$2Cl^-$ cotransporter 1
NMDA	N-methyl D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAD	Primary afferent depolarisation
PAF	Primary afferent fibres
PAM	Positive allosteric modulators
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PIER	Protease-induced epitope retrieval
PLP	Pyridoxal phosphate
PNS	Peripheral nervous system
PRRs	Pattern-recognition receptors
QOL	Quality of life
qPCR	Quantitative PCR

qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SD	standard deviation
siRNA	Small interfering RNA
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SSA	Succinic semialdehyde
SSADH	Succinic semialdehyde dehydrogenase
TBE	Tris-borate-EDTA
TCA	Tricyclic acid
TE	Tris-EDTA
TLR	Toll-like receptors
TMJ	Temporomandibular joint
TRG	Trigeminal ganglion
TRP	Transient receptor potential
VDCCs	Voltage-dependent calcium channels
VGAT	Vesicular GABA transporters
VGCCs	Voltage-gated Ca^{2+} channels
β-ME	B-Mercaptoethanol

LIST OF APPENDICES

EKPRESI NEUROTRANSMITER RENCATAN RESEPTOR $GABA_A$ DALAM PULPA GIGI MANUSIA: PERANAN DAN POTENSI DALAM LALUAN SAKIT GIGI

ABSTRAK

Sakit berkaitan struktur gigi adalah keadaan lazim yang boleh menjejaskan kehidupan seharian seseorang. Sakit ini boleh terjadi akibat penyakit pergigian mahupun ketika menjalani rawatan pergigian. Perkembangan perawatan dalam sakit gigi ini sangat mencabar berikutan mekanisme isyarat sakit dalam pulpa gigi yang masih tidak jelas asid gamma-aminobutirik (GABA) ialah neurotransmiter perencat utama dalam sistem saraf pusat mamalia dan mempunyai peranan dalam mekanisme isyarat kesakitan. Beberapa kajian ke atas GABA dan reseptornya dalam pulpa gigi telah dijalankan namun, peranan sebenar reseptor ini dalam mekanisme isyarat sakit gigi masih belum difahami sepenuhnya. Kajian ini dijalankan untuk menyiasat ekspresi gen dan protein bagi dua subunit reseptor $GABA_A$ yang paling kerap dilaporkan, α1 dan β2, dalam pulpa gigi manusia yang sihat. Untuk mencapai objektif kajian, teknik seperti pengasingan RNA, tindak balas rantai transkripsi-polimerase terbalik (RT-PCR) dan imunohistokimia (IHC) telah digunakan. Sebelum melakukan RT-PCR, suhu penyepuhlindapan untuk proses amplifikasi optimum *GABRA1* dan *GABRB2* didapati 55°C. Analisis ekspresi gen menggunakan RT-PCR menunjukkan kehadiran *GABRA1* dan *GABRB2* dalam pulpa gigi, dan analisis ujian T sampel tak bersandar menunjukkan bahawa ekspresi *GABRA1* adalah lebih tinggi secara signifikan dari *GABRB2*. Pewarnaan imunohistokimia memberikan bukti visual ekspresi protein GABRA1 dan GABRB2 dalam lapisan odontoblast pulpa gigi, menunjukkan kehadirannya dalam sel badan dan proses odontoblastik yang mengunjur

ke dalam dentin. Hasil pewarnaan IHC yang optimum diperoleh dengan menggunakan penimbal Tris-EDTA (TE) pada pH 9 untuk prosedur mendapatkan semula antigen dengan kepekatan antibodi 1:50 dan 1:200 masing-masing untuk antibodi GABRA1 dan GABRB2. Penemuan ini menyokong hipotesis bahawa subunit reseptor $GABA_A$ α1 dan β2 diekspreskan dalam pulpa gigi manusia. Ekspresi gen dan protein subunit ini memberi gambaran yang lebih jelas dalam memahami dan menjalan kajian selanjutnya mengenai peranan $GABA_A$ subunit α1 dan β2 dalam mekanisme isyarat sakit berkaitan pergigian. Kajian-kajian seterusnya yang menggunakan sampel berpenyakit dan penyiasatan dari aspek fungsian amat wajar untuk dilaksanakan bagi mengkaji dengan lebih terperinci mekanisme dan kesan sebenar $GABA_A$ subunit α1 dan β2 dalam sakit gigi serta potensinya dalam rawatan sakit berkaitan gigi.

EXPRESSION OF INHIBITORY NEUROTRANSMITTER $GABA_A$ RECEPTORS IN HUMAN DENTAL PULP: A POTENTIAL ROLE IN DENTAL NOCICEPTIVE SIGNALLING

ABSTRACT

Dental pain is a prevalent and distressing condition that can have a significant impact on a person's daily life. It can arise from various causes, including dental diseases and conditions, as well as during dental treatments. Development of effective pain management strategies in the dental setting remains a challenge due to the unclear mechanisms of pain signalling in the dental pulp. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS) and has a well-established role in pain signalling. While several studies have explored the expression GABA and its receptors in the dental pulp, the exact influence of these receptors on dental pain signalling is still not fully understood. Therefore, this study aimed to investigate the gene and protein expression of the two most abundantly expressed $GABA_A$ receptor subunits, α1 and β2, in the healthy human dental pulp. In order to achieve the objective of the study, techniques such as RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) were employed. Prior to performing RT-PCR, the annealing temperature for amplification of both target genes were found to be 55°C. Gene expression analysis using RT-PCR demonstrated the presence of *GABRA1* and *GABRB2* in the dental pulp, and independent T-test analysis indicated that the expression of *GABRA1* was significantly higher than *GABRB2*. Immunohistochemical staining provided visual evidence of GABRA1 and GABRB2 protein expression in the odontoblast layer of dental pulp, indicating their presence in cell bodies and odontoblastic processes

extending into the dentin. Optimal IHC staining results were obtained by using Tris-EDTA (TE) buffer at pH9 for antigen retrieval with antibody concentrations of 1:50 and 1:200 for the GABRA1 and GABRB2 antibodies, respectively. These findings support the hypothesis that $GABA_A$ receptor α1 and β2 subunits are expressed in human dental pulp. The presence of gene and protein expression of these subunits offers valuable insights for further research into the potential roles of $GABA_A$ receptors in dental related pain signalling. Future studies using diseased samples and functional investigations are warranted to explore the precise mechanisms and implications of $GABA_A$ receptor α1 and β2 subunits in dental pain and their potential therapeutic applications.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Dental pain is a common and often debilitating condition that affects millions of people worldwide and is one of the most common reasons for individuals to seek dental care (World Health Organization, 2022). It is defined as “pain caused by lesions or disorders affecting one or more teeth and/or immediately surrounding and supporting structures: the tooth pulp, periodontium and gingivae” (“International Classification of Orofacial Pain, 1st edition (ICOP),” 2020). Dental pain can arise due to a variety of factors, including tooth decay, periodontal disease, infection or trauma and the intensity and duration can vary widely, ranging from a mild discomfort to a sharp, throbbing pain that can interfere with daily activities. The Global Burden of Disease Study reported that the global prevalence of oral conditions in 2017 was approximately 3.5 billion people (Bernabe *et al.,* 2020). The effects of dental pain extend beyond the physical discomfort experienced by individuals. It has a substantial impact on various aspects of an individual's life, including their quality of life, social and financial well-being. Dental pain can significantly impair a person's ability to comfortably eat, speak and perform daily activities (Cavalheiro *et al.*, 2016). This may cause embarrassment and reduced self-confidence, leading to social isolation and a negative impact on their social relationships (Salvador & Toassi, 2021). Additionally, the financial burden associated with dental pain, including costs of dental visits, treatments and potential loss of productivity due to pain-related limitations, further exacerbates the issue (Aldosari *et al.*, 2021).

The most common cause of dental pain is tooth decay, also known as dental caries or dental cavities. In 2017, the number of prevalent cases of caries in permanent

teeth was reported to be 2.3 billion (Bernabe *et al.*, 2020). Dental carries are the result of demineralisation of the enamel that occurs due to the acid produced by bacteria buildup in the mouth. As the hard protective outer layer of enamel and dentine is eroded away, this exposes the inner layers of the tooth i.e. the pulp, thus causing pain and discomfort. Other than pulpal exposure, exposure of the dentin can also cause dental pain in a condition known as dentinal hypersensitivity (DH) (Liu *et al.*, 2020). When the protective enamel layer is worn down or when gum tissue recedes, the dentin is exposed to external factors such as hot or cold temperatures, sweet foods or drinks, or even brushing and flossing which can cause pain.

Treatment for dental pain depends on the underlying cause and severity of the pain. In cases of caries without pulpal exposure, treatment might include a tooth filling where the decay is cleaned and a protective material, such as composite resin or amalgam, is placed in the cavity to restore the tooth's structure and prevent further decay. However, in cases of extensive caries where the pulp has been exposed, endodontic treatments such as root canal therapy might be required. During a root canal, the infected dental pulp is removed, the canals are cleaned and filled and the tooth is then sealed to prevent further infection (Pietrzycka *et al.*, 2022). For dental pain associated with cases of periodontal disease, deep cleaning and antibiotics may be prescribed to treat the infection and prevent further damage to the gums and teeth. In cases of cracked or broken teeth, a dental crown or bonding may be necessary to restore the tooth's structure and prevent further damage.

Pain management during dental procedures is an important aspect of dental care, as the fear of pain during and after treatments tend to discourage patients from dental visits. Delayed treatment may then lead to other problems, such as infections

and inflammation which might eventually lead to a treatable tooth needing to be extracted. As tooth extraction has its own set of problems that have been associated with a negative impact on the quality of life, better management of pain during dental procedures is crucial to ensure that patients are not deterred from seeking required dental treatments (Wigsten *et al.*, 2020). The most common method of intraoperative pain management is the administration of local anaesthesia (Wang *et al.*, 2021). It is injected prior to the dental procedure to cause temporary sensory loss at the site of injection. This allows the dentist to carry out the treatment with minimal discomfort to the patient. In addition to intraoperative pain, a common concern in patients undergoing invasive treatments such as root canals and tooth extractions is postoperative pain. While soreness and pain after a dental procedure is normal, it is important to manage it appropriately to prevent further complications and ensure a smooth recovery. Drugs that are usually prescribed for managing postoperative dental pain may include nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen and opioids (Zanjir *et al.*, 2020).

While these drugs are commonly used for dental pain management, it is crucial to acknowledge their limitations which include potential side effects, adverse drug interactions and the risk of abuse. An example of a limitation seen with NSAIDs, which are often prescribed as the initial treatment for dental pain due to their high efficacy, is the notable risk of gastrointestinal toxicity (Sostres *et al.*, 2010). NSAIDs are also contraindicated in individuals with nephropathy, pre-existing conditions of the gastrointestinal mucosa, haemorrhagic disorders, or for those undergoing anticoagulant therapy (Becker, 2010). For such patients, a nonopioid alternative that is frequently prescribed is acetaminophen, which is commonly known as paracetamol. However, due to the metabolism of acetaminophen in the liver, hepatotoxicity is the

most significant adverse effect. While generally safe within the recommended dosage of less than 4 grams per day, this dosage may pose a risk of toxicity for individuals with alcoholism, malnutrition or pre-existing chronic liver disease (Hargreaves *et al.*, 2005). In certain cases, dentists may prescribe opioids in combination with NSAIDs and acetaminophen for dental pain management. This is usually done due to the lack of nonopioid alternatives when NSAIDs and acetaminophen are contraindicated or ineffective. Due to the potential risk of misuse, dependency or addiction, as well as the adverse effects of opioids such as drowsiness, respiratory depression, nausea and vomiting, opioids are usually not considered as first choice of analgesics (Schroeder *et al.*, 2019).

Although there have been continuous efforts to understand the cellular and molecular mechanisms involved in dental pain, their exact mechanisms underlying dental pain are still not completely understood. This lack of understanding has contributed to the difficulty in developing effective pain management strategies. However, in recent years, various ion channels and receptors, including the transient receptor potential (TRP) channels, have been identified to play a role in dental pain signalling (Hossain *et al.*, 2019). The TRP channels are a family of non-selective ion channels present in dental primary afferent neurons and odontoblasts that are believed to play a role in transducing different types of external stimuli involved in dental pain. TRP channels have emerged as a novel therapeutic target for managing dental pain. Several preclinical and clinical studies have shown that TRPV1 receptor agonists have significant analgesic effects and with further research, effective and safe TRPV1 targeted therapies for dental pain management can be development (Gavva *et al.*, 2008; Badral *et al.*, 2013; Quiding *et al.*, 2013).

Given the extensive research conducted on TRP channels and their established role in dental pain signalling, which has facilitated the development of targeted drugs, it is probable that a similar approach can be undertaken for other ion channels present in the dental pulp. Thus this study aims to investigate the presence of gamma-aminobutyric acid type A receptor subunit alpha 1 (GABRA1) and gamma-aminobutyric acid type A receptor subunit beta 2 (GABRB2) in the human dental pulp. These subunits are the two most common subunit that make up the $GABA_A$ receptor (Ghit *et al.*, 2021) . $GABA_A$ receptors are pentameric ligand-gated ion channels that play a crucial role in mediating fast synaptic transmissions and inhibitory neurotransmission in the central nervous system (CNS). These receptors are activated by gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter that has been established as a key player in pain signalling within the central nervous system. By further understanding GABAergic mechanisms in dental tissues, novel interventions and drugs that enhance GABAergic activity or target specific GABA receptors can be developed, paving the way for effective pain relief strategies by reducing neuronal excitability and inhibiting pain transmission. GABAergic drugs, including drugs that target GABA receptors such as benzodiazepines, as well as GABA derivative which includes gabapentin and pregabalin are being studied for pain management in various diseases (Vuilleumier *et al.*, 2013; Du *et al.*, 2022). By studying and improving the understanding of GABAergic signalling in the dental tissues, new therapeutic approaches and GABAergic specific interventions can be explored. By developing drugs that enhancing GABAergic activity or targeting specific GABA receptors, effective pain relief strategies can be investigated.

1.2 Justification of the study/Problem statement

The presence of functional $GABA_A$ receptors with the α1β2γ2 subunit combinations have been studied in various tissues, including the brain, retina, spinal cord and dorsal root ganglia (DRG) (Sigel et al., 2012; Du et al., 2017). However, there is a lack of literature regarding these subunits in relation to the dental pulp. Despite continuous advancements and ongoing research to understand pain signalling in the dental pulp, the exact mechanism still remains uncertain. Identifying the specific molecular targets involved in dental pulp pain pathway would open up new possibilities for treating dental pain. As the pentameric $GABA_A$ receptor 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. Since each subunit contributes to their distinct pharmacological and physiological properties, receptors composed of different subunit combinations can exhibit variations in their response to drugs and compounds that target GABAergic signalling. For example, benzodiazepines are a class of drugs that has been known to allosterically modulate $GABA_A$ receptors by binding between the α and γ subunits. However, these drugs show a higher affinity for receptors with α1 subunits as compared to α2 or α3 subunit, and they do not have an effect on $GABA_A$ receptors with α5 subunits (Olsen, 2018a). This variations in drug affinity and pharmacological response highlights the importance of considering the subunit composition of $GABA_A$ receptors when studying their pharmacology and developing drugs that target GABAergic signalling. Identifying the subunit composition of $GABA_A$ receptors in the dental pulp can help in designing drugs that selectively target certain receptor isoforms while avoiding or

minimizing interactions with others subunits that may be associated with undesired outcomes (Ghit *et al.*, 2021).

1.3 Research question

Are $GABA_A$ receptor α1 and β2 subunits expressed in the human dental pulp?

1.4 Research objectives

1.4.1 General objective

To investigate the gene and protein expression of $GABA_A$ receptor α1 and β2 subunits in the human dental pulp.

1.4.2 Specific objectives

1. To determine and compare the gene expression of $GABA_A$ receptor α1 (*GABRA1*) and β2 (*GABRB2*) subunits in human dental pulp via reverse-transcription polymerase chain reaction (RT-PCR).
2. To compare the gene expression of *GABRA1* and *GABRB2* in human dental pulp using statistical analysis.
3. To determine the protein expression of GABRA1 and GABRB2 in human dental pulp via immunohistochemical fluorescence staining.

1.5 Research hypotheses

The hypothesis of this study is that $GABA_A$ receptor α1 and β2 subunits are expressed in human dental pulp.

CHAPTER 2

LITERATURE REVIEW

2.1 Pain and nociception

In 1996 the International Association for the Study of Pain (IASP) defined pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage’. This definition was then revised in 2020 and currently states that ‘pain is an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage’ (Raja *et al.*, 2020). It was also noted that the terms pain and nociception describe different phenomenon which are not interchangeable. While nociception is the process by which noxious stimulus are processed and transmitted throughout the nervous system and can be observed from a third person's perspective, pain is a subjective experience that can only be expressed by the person who feels it (Treede, 2018). Although in most cases nociceptive stimulation does lead to pain, nociception and pain can exist independently of each other (Nickel *et al.*, 2017).

2.1.1 Process of nociception

The process of nociception begins with the activation of the free nerve endings present in sensory neurons. These sensory nerve fibres can be classified into different types based on their myelination, diameters and conductance velocities as depicted in Table 2.1. Nociceptive fibres can be classified as myelinated Aδ or unmyelinated C fibres. Aδ fibres detect thermal or mechanical stimuli while C fibres detect thermal, mechanical and chemical stimuli. Due to the myelination of Aδ fibres, they conduct impulses faster than C fibres, producing a rapid response of sharp localised pain whereas the thinner unmyelinated C fibres transmit impulses that result in slow and dull pain (Dubin *et al.*, 2010). These sensory neurons are primary afferent nerve fibres which

have their cell bodies either in the DRG or trigeminal ganglion (TRG). They are pseudounipolar cells with an axon that emanates from the cell body which then bifurcates, allowing the peripheral branch to innervate the skin while the central branch leads to the spinal cord (Krastev *et al.*, 2013).

Table 2.1 Classification of nociceptive fibres.

Fibre Type	**Myelination**	**Diameter (µm)**	**Conduction Velocity (m/s)**	**Receptors present**	**Sensory Information Conveyed**
Aα	Myelinated	12-20	70-120	Proprioceptors	Proprioception
Aβ	Myelinated	5-12	20-707	Proprioceptors and mechanoreceptors	Discriminative touch and pressure
Aδ	Myelinated	1-5	5-30	Nociceptors and thermoreceptors	Nociception: Sharp pain Thermal: cold detection
C	Unmyelinated	0.1-1.3	0.6-2.0	Nociceptors and thermoreceptors	Nociception: dull, aching pain Thermal: heat detection

*Adapted from (Maslinska *et al.*, 2018).

The peripheral terminals of these neurons contain numerous receptors and ion channels including voltage-gated sodium, potassium and calcium channels, TRP channels, acid-sensitive cation channels (ASIC), hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels, purinergic P2X receptors, 5-HT3 serotonin receptors and various G-protein-coupled receptors (GPCRs) that indirectly activate ion channels (Raouf et al., 2010; Benarroch, 2015). Sufficient noxious stimuli activate these receptors causing generation of action potentials. These action potentials are electrical signals that are transmitted to the spinal cord via DRG, then to the brain via the spinothalamic tract (Lingford-Hughes *et al.*, 2012).

The neurons entering the spinal cord ascend or descend a few vertebral levels via Lissauer's tract before entering the dorsal horn where the central end of Aδ fibres project to laminae I and V while the central end of C fibres project to laminae I and II. In the dorsal horn, the primary afferent fibres synapse with second order neurons. The axons of these second order neurons decussate through the ventral white commissure and ascend upwards through the brain stem and synapse onto third order neurons in the thalamus. Third order neurons then send the signal to the primary somatosensory cortex where the signal is perceived and interpreted (Lingford-Hughes *et al.*, 2012).

2.2 Dental pain

Dental pain is a subcategory of orofacial pain attributed to disorders of dentoalveolar and anatomically related structures. Dental pain is defined as "pain caused by lesions or disorders affecting one or more teeth and/or immediately surrounding and supporting structures: the tooth pulp, periodontium and gingivae" ('International Classification of Orofacial Pain, 1st edition (ICOP)', 2020). The evident relationship between dental pain and oral diseases such as dental caries, periodontal

disease, pulpitis or dentoalveolar trauma has been well established (Fukuda, 2016). The common presentation of these conditions is the exposure of the dentin or pulp which may cause DH or inflammation of the dental pulp leading to inflammatory pain.

Several studies have reported that pain was the main reason for visiting the dentist (Daou *et al.*, 2016; Murshid, 2016; Rambabu *et al.*, 2018; Kamalova *et al.*, 2020). This is most probably due to the negative impact of dental pain on a person's quality of life (QOL). Talking, eating and sleeping are among the main daily activities that are affected by dental pain (Cavalheiro *et al.*, 2016). Among school going adolescents, dental pain was associated with an increased school absenteeism, decreased attention span, inability to concentrate, poorer school performance and lack of interest in playing and socializing with others (de Paula *et al.*, 2013; Rebelo *et al.*, 2019; Santos *et al.*, 2019).

2.2.1 Structure of the tooth

Teeth are composed of four different tissues: enamel, cementum, dentin and dental pulp. Enamel, dentin and cementum are hard mineralised tissues which encapsulate the soft innervated dental pulp. Enamel is found on the crown of the tooth and plays a role in mastication as well as protecting the dentin and pulp from injury while cementum, present on the surface of the root, is responsible for anchoring the tooth to the alveolar bone. Dentin is present between the enamel and dental pulp and is responsible for maintaining the structural stability of the tooth. Dentin is made up of numerous microtubules known as dentinal tubules which are filled with fluid. The dental pulp is the only soft tissue in the tooth and is highly innervated and vascularised (Tjäderhane *et al.*, 2019).

The dental pulp is innervated by sensory trigeminal afferent neurons that enter the tooth via a small opening at the tip of the root known as the apical foramen. These nerves enter the apical foramen as two or three thick bundles of myelinated Aδ fibres alongside several smaller nerve bundles. The thick bundle of nerves travel fairly straight through the root pulp reaching the coronal pulp, while the smaller nerve bundles innervate the root pulp. In the coronal pulp, the larger bundles begin to form branches which arborize extensively forming an intricate network of interwoven nerves known as the plexus of Raschkow (Nair *et al.,* 1992). In multi rooted teeth, the branches entering via the different roots were responsible for innervating the corresponding pulpal horn. These branches tend to travel further into the horn before ramifying and terminating as free nerve endings near or on the odontoblastic cells layer (Engström *et al.*, 1960).

The sensory nerve fibres that are present in the dental pulp are the unmyelinated C fibres and myelinated A fibres (Aβ and Aδ fibres) (Miyoshi *et al.*, 1966; Allison *et al.*, 2020). Based on electron microscopy results, myelinated fibres only account for 13%, while unmyelinated fibres constitute the remaining 87% (Walton *et al.,* 1995; Zhan *et al.,* 2021). While 90% of the A fibres are thinly myelinated Aδ fibres which are responsible for nociception, the remaining 10% are the highly myelinated Aβ fibres, which indicate a possible mechanoreceptive function in the dental pulp (Nair *et al.*, 1992; Jain *et al.*, 2013). The Aδ fibres are mainly present at the pulp dentin border of the coronal pulp and are concentrated in the pulp horns. The C fibres are located in the core of the pulp and extend into the cell-free zone underneath the odontoblastic layer (Davies *et al.*, 2019).

2.2.2 Dental pain pathway

As dental pain falls under the subcategory of orofacial pain, it is processed by the trigeminal nervous system (Figure 2.1). The trigeminal nerve, also known as the fifth cranial nerve (CN V), is responsible for detecting sensations in the face. The CN V divides out into three branches: ophthalmic (V1), maxillary (V2) and mandibular (V3). The ophthalmic branch sends impulses from the upper part of the face including the eyes, forehead and scalp to the brain. The maxillary branch detects sensation in the middle part of the face including the nose, cheeks, upper jaw while the mandibular branch detects sensation from the lower jaw, lower lips and gums. These three branches converge to form the TRG which houses the cell bodies of the sensory neurons of the trigeminal nerve (Huff et al., 2022).

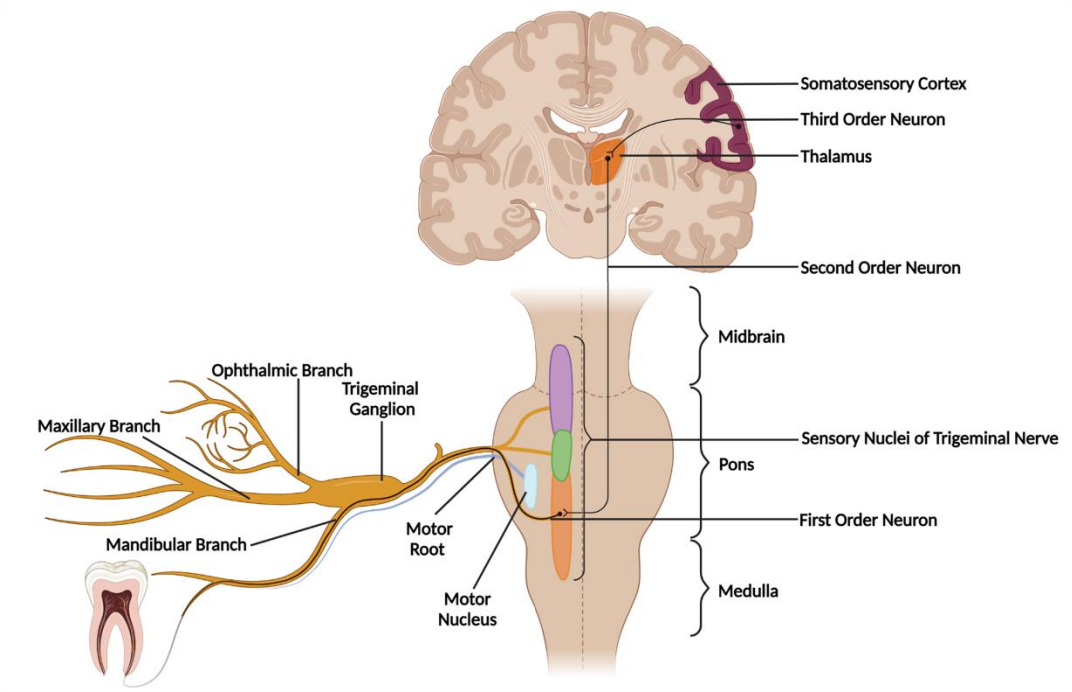

Figure 2.1 Schematic representation of the dental pain pathway. The pain signals originating from the teeth travel via the maxillary and mandibular branches of the trigeminal nerve, through the trigeminal ganglion and reach the sensory nuclei of the trigeminal nerve. In the sensory nucleus, the first order neuron, synapses with the second order neuron, enabling the transmission to progress to the thalamus. At the thalamus, the second order neuron synapses on the third order neuron, which then projects to the primary somatosensory cortex in the brain, where pain is perceived and localised (Sivakumar et al., 2022).

All sensory fibres from the CN V terminate in the trigeminal nucleus located at the lateral medulla of the brain stem. The trigeminal nucleus is divided into three sensory nuclei: the mesencephalic nucleus, the main sensory nucleus and the spinal trigeminal nucleus. The mesencephalic nucleus is responsible for processing proprioception from the jaw while the main sensory nucleus is responsible for processing touch-pressure sensations from the facial region. The spinal trigeminal nucleus receives information about pain and temperature sensations respectively and is further divided into three parts: pars oralis, pars caudalis and pars interpolaris. Pars oralis and pars interpolaris are involved in transmission of discriminative tactile sensations from the face while the pars caudalis is responsible for transmission of nociceptive and thermal sensations (Huff *et al.*, 2022).

During dental nociception, the free nerve endings of myelinated Aδ fibres and unmyelinated C fibres in the dental pulp are activated. The impulses then travel along first order neurons, through the TRG, to reach the pars caudalis of the spinal trigeminal nucleus. Within the pars caudalis, first order neurons then synapse onto second order neurons. These secondary neurons then transmit the signals through the trigeminothalamic tract, carrying the information towards the thalamus. Here, the secondary order neurons establish connections with third order neurons, which then convey the impulses to the primary somatosensory cortex of the brain for further processing (Lingford-Hughes *et al.*, 2012).

2.2.3 Common causes of dental pain

2.2.3(a) Inflammation of the pulp

Inflammation of the dental pulp, also known as pulpitis, usually occurs due to bacteria that enter the pulp, either via dental caries, cracks in the tooth or in rare cases, via the apical foramen in cases of periodontitis (Gautam *et al.*, 2017). Dental caries are

the most common oral disease reported by The Global Burden of Disease Study 2017. Globally, in 2019, prevalent cases of caries of permanent teeth and primary teeth were 2.03 billion and 0.52 billion respectively (Qin *et al.*, 2022). Dental caries, also known as tooth decay, occurs due to the demineralisation of the hard tissues of the tooth. This destruction is caused by the acid that is produced by bacteria in the oral cavity that feed on sugar and starch (Karpiński *et al.*, 2013). As the acid continues demineralizing the enamel, a carious lesion is formed and it progressively deepens, eventually reaching the dentin. As these cariogenic bacteria proliferate, they release cell wall components such as lipoteichoic acid and lipopolysaccharides that diffuse through dentinal tubules and move towards the dental pulp (Jiang *et al.*, 2006). These molecules are known as pathogen-associated molecular patterns (PAMPs) and are molecular structures shared by various pathogens essential for microorganism survival (Akira *et al.*, 2006). These molecules are the early inducers of caries-related pulp inflammation.

Due to their localisation at the pulp-dentin interface, as well as their long projections into the dentinal tubules, odontoblasts are the first pulpal cells that encounter these PAMPs. Thus odontoblasts are seen as the first line of defence and contain several pattern-recognition receptors (PRRs) in their cell membrane. One of the major classes of PRRs present in odontoblasts are toll-like receptors (TLRs), which are responsible for triggering the first line of immune defence known as innate immune response (Veerayutthwilai *et al.*, 2007). This is achieved by the upregulation of molecular effectors of innate immunity, such as antimicrobial peptides as well as proinflammatory cytokines and chemokines (Farges *et al.*, 2015). These inflammatory mediators cause the activation and sensitisation of intraductal A and C fibres. As A fibres have myelinated axons allowing for a fast conduction speed, these are the first fibres that transmit pain signals resulting in rapid and sharp pain that is reported by patients with

acute pulpitis. As the inflammation reaches further into the centre of the pulp, more C fibres are activated, causing a dull and lingering type of pain, characteristic of chronic pulpitis, to develop. Worsening of the inflammation also causes the pulp to become hypoxic and as A fibres have much higher oxygen consumption then the C fibre, the latter are more tolerant to the conditions and continue to transmit pain signals for a much longer time.

2.2.3(b) Dentine hypersensitivity (DH)

Other than the highly innervated dental pulp, nerve fibres have also been found to penetrate the odontoblast layer and enter the dentinal tubules (Byers *et al.*, 1982). These fibres are mainly Aδ fibres and C fibres which respond to both mechanical and thermal stimuli. This is the reason for dental pain that occurs in the absence of pulp exposure. This condition is known as DH and has been defined as "pain derived from exposed dentin in response to chemical, thermal tactile or osmotic stimuli which cannot be explained as arising from any other dental defect or disease" (Hypersensitivity, 2003). The primary cause of DH is exposure of the dentin due to the loss of enamel or gum recession thus exposing both coronal and root dentin. Patients with DH often complain of sharp pain induced by non-noxious stimuli such as touch, air-flow or while consuming food or drinks that are mildly hot, cold or sweet (Holland *et al.*, 1997). As opposed to inflammatory pain, pain caused by DH tends to disappear as soon as the external stimulus in removed.

Three main mechanisms of DH have been proposed. The first one being the neural or direct innervation theory which suggest that the nerve endings present in the dentinal tubules are capable for detecting the noxious stimuli and directly transmit pain signals to the trigeminal ganglion. While there have been studies that report the presence

of nerve fibres in the root dentine, there is little evidence of nerve fibres in the superficial dentin to prove this theory (Frank *et al.*, 1988). The second theory, known as the odontoblast transducer theory states that odontoblasts play a role as sensory transducers. While some studies report that there are no synapses or neurotransmitters present for signalling to be possible between odontoblast and pulpal nerve fibres (Bartold, 2006), there have also several studies providing evidence that odontoblasts may have a sensory function. The expression of functionally active thermo-sensing ion channels such as TRP channels on odontoblast cells (El Karim *et al.*, 2011a; Tazawa *et al.*, 2017) and the possibility that even in the absence of a neural synapse, paracrine signalling may be responsible for transmitting signals from the cells to adjacent nerves provide evidence for this theory (Liu *et al.*, 2020). The last and most widely accepted theory is the hydrodynamic theory which proposes that the thermal or mechanical stimuli causes movement of the dentinal fluids which triggers the activation of nociceptors present in the pulp dentin border (Brännström *et al.*, 1964). The flow of the dentinal fluid can be inward towards the pulp, or outward towards the dentine based on the stimuli. Cooling or dehydration of the dentin surface causes the outward flow of fluid while heating or mechanical pressure seem to cause the inward flow of fluid (Orchardson *et al.*, 2001). The movement of fluid in the tubules is thought to activate the nerve endings of Aδ fibres resulting in the short, sharp pain characteristic of DH (Matthews *et al.*, 1994). The presence of molecular mechnotransducers such as the TRP channels on dental afferents also provides evidence for the mechanical detection of dentinal fluid movement (Chung *et al.*, 2013).

2.3 Gamma-aminobutyric acid (GABA)

GABA is a naturally occurring amino acid that plays a role as the major neurotransmitter involved in synaptic transmission throughout the mammalian CNS. GABA exerts its inhibitory effects by binding to transmembrane GABA receptors present on the plasma membrane of postsynaptic neurons and hyperpolarizing them. As a result, the firing of an action potential is inhibited (Kaur *et al.*, 2020).

2.3.1 GABA synthesis

GABA is synthesised in inhibitory neurons via a closed loop metabolic pathway known as the GABA shunt (Figure 2.2). It begins with the enzyme glutamate dehydrogenase which is responsible for the transamination of α-ketoglutarate (from the tricyclic acid (TCA) cycle) into glutamate. Glutamate is then decarboxylated to form GABA. This is an irreversible and rate limiting reaction which is catalysed by the enzyme glutamate decarboxylase (GAD) using pyridoxal phosphate (PLP) as a cofactor. In the brain, two forms of the GAD enzyme have been reported: GAD67 and GAD65. These two isoforms are encoded by the GAD1 and GAD2 genes respectively. While GAD67 is expressed throughout the neuron, GAD65 has been found to be localised to the neuronal terminals. This is due to the fact that GAD65 synthesises GABA only for neurotransmission while GAD67 synthesises GABA during synaptogenesis or for protection from neural injury (Feldblum *et al.*, 1993). GABA is then catabolised by GABA transaminase (GABA-T) producing succinic semialdehyde (SSA) which is further converted to succinate by the enzyme succinic semialdehyde dehydrogenase (SSADH). Succinate then re-enters the TCA cycle and completes the looped process of the GABA shunt (Kleppner *et al.*, 2002)

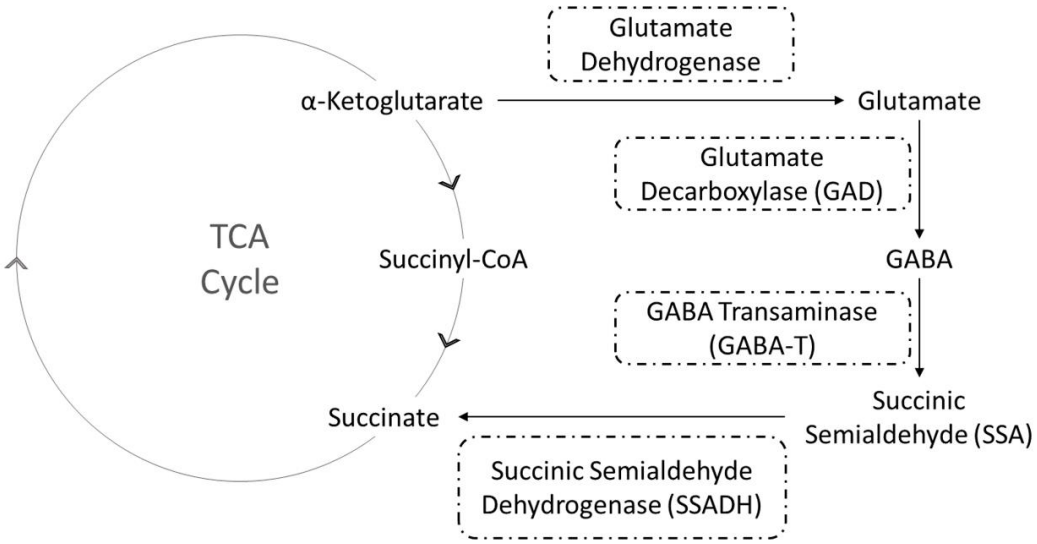

Figure 2.2 GABA synthesis via the GABA shunt. GABA synthesis begins with the transformation of α-ketoglutarate into glutamate by glutamate dehydrogenase. Then, in the process of glutamate decarboxylation, which is facilitated by GAD, glutamate is converted into GABA. GABA is further metabolised by GABA-T to produce SSA which is then converted back to succinate by SSD, thus allowing succinate to re-enter the TCA cycle and complete the looped process which is known as the GABA shunt. (GABA: gamma-aminobutyric acid; GAD: glutamate decarboxylase; GABA-T: GABA transaminase; SSA: succinic semialdehyde; SSADH: succinic semialdehyde dehydrogenase, TCA: tricyclic acid). (Sivakumar *et al.*, 2022).

2.3.2 GABA release

Once synthesised in the neuron, GABA is packaged into vesicles via vesicular GABA transporters (VGAT) and stored at the terminals of GABAergic neurons (McIntire *et al.,* 1997). When the neuron gets depolarised due to an action potential, the membrane potential changes and this causes voltage-dependent calcium channels (VDCCs) to open. The increase in calcium concentration in the axon stimulates GABA containing synaptic vesicles to move towards the cell membrane of the neuron terminals. The vesicles then fuse with the cell membrane causing GABA to be exocytosed. GABA then diffuses across the synaptic cleft and binds to its receptors on the post synaptic neuron (Chaudhry *et al.*, 1998).

2.3.3 GABA reuptake

Once GABA binds to the receptors on the post synaptic membrane and the action potential has been transmitted, GABA needs to be cleared from the synaptic cleft in preparation for the next action potential. This process is modulated by GABA transporters (GATs) which transport GABA back into presynaptic neurons or surrounding glial cells. As GATs belong to the superfamily of Na^+ and Cl^- dependent transporters, they use the energy from the steep transmembrane sodium gradient to actively transport the extracellular GABA back into cells (Ransom *et al.*, 2009). GABA molecules that are taken up by the presynaptic neurons are repackaged into vesicles by VGAT and are ready to be released during the next action potential. However, GABA that is taken up by the glial cells cannot be reused and instead are metabolised, eventually ending up as glutamine which is transported back to the presynaptic neurons and reused for GABA synthesis (Brambilla *et al.,* 2003).

2.3.4 GABA receptors

GABA binds and activates two types of receptors: ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. $GABA_A$ receptors are responsible for mediating faster synaptic transmission, while $GABA_B$ receptors mediate slower synaptic transmission.

2.3.4(a) $GABA_A$ receptors

$GABA_A$ receptors are pentameric ligand-gated ion channels with a central Cl^- ion channel. The receptor comprises of five transmembrane polypeptide subunits, each with one extracellular N-terminal domain, four hydrophobic transmembrane domains (Ml-M4) and one extracellular C-terminal domain. These subunits form a cylindrical structure with the M2 region of each subunit lining the pore (Cherubini *et al.*, 2001). This motif is also seen in other pentameric ligand-gated receptors including nicotinic acetylcholine receptors, zinc-activated ion channels and glycine receptors (Nemecz *et al.*, 2016).

In mammals, there are 19 different $GABA_A$ subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3) and $GABA_A$ receptors can be assembled with varying subunits. Each subunit is encoded by a different gene and even splicing variants have been identified (Whiting *et al.*, 1990). Due to the many subunits, a number of potential subunit combinations can be obtained. However, various studies have concluded that functional $GABA_A$ receptors contain at least one α, one β and one other subunit (Baumann *et al.*, 2002). This is mainly due to the GABA binding site being located at the interface between the α and β subunits. While majority of $GABA_A$ receptors contains two α subunits, two β subunits and one γ subunit, variation in the subunit subtypes provide different variations of the 2α:2β:1γ conformation. $\alpha_2\beta_2\gamma_1$ has been reported to be the major subtype, making up 60% of all $GABA_A$ receptors and found synaptically and

extra-synaptically. α2β3γ2 and α3βnγ2, two other minor subtypes that are commonly observed, represent (15–20%) and (10–15%) of $GABA_A$ receptors respectively (Möhler, 2006). The subunit composition of GABA receptors have been shown to have an effect on the functional and pharmacological properties as well as the affinity and sensitivity of the receptor to GABA (Verdoorn *et al.,* 1990; Krogsgaard-Larsen *et al.,* 2002; Rudolph *et al.,* 2004).

The binding of GABA on post synaptic $GABA_A$ receptors causes the opening of the Cl^- channel, allowing Cl^- ions to enter the neuron. As this decreases the membrane potential and hyperpolarises the post synaptic neuron, decreasing the possibility that the neuron will fire an action potential. It is in this way that GABA exerts its inhibitory effects via $GABA_A$ receptors (Kleppner *et al.,* 2002).

Other than GABA, various other molecules that can bind to and regulate $GABA_A$ receptor activity have been reported. These other ligands may bind to the orthosteric site (i.e. where GABA binds) or on other allosteric sites. While muscimol, gaboxadol and progabide are orthosteric agonists whose binding causes activation of the receptor, orthosteric antagonists such as bicuculline, picrotoxin and gabazine competitively inhibit GABA from binding. (Bartholini, 1984; Vashchinkina *et al.,* 2012; Johnston, 2013), On the other hand, allosteric modulators exert their effect by binding to other sites on $GABA_A$ receptors. Benzodiazepines, barbiturates and ethanol are examples of positive allosteric modulators (PAM) while pregnenolone sulfate and zinc are examples of negative allosteric modulators (NAM) (Olsen, 2018b; Vega Alanis *et al.,* 2020).

2.3.1(a)(i) GABRA1

The $GABA_A$ receptor α1 subunit is encoded by the *GABRA1* gene and is mapped to a cluster of genes on chromosome 5q34 encoding α1, α6, β2 and γ2 subunits of the $GABA_A$ receptor (Ghit *et al.*, 2021). As discussed above, all $GABA_A$ receptor subunits have a similar structure with an extracellular N-terminal domain, four hydrophobic transmembrane domains (Ml-M4) and one extracellular C-terminal domain (Zhang *et al.*, 2022).

GABRA1 expression has been found to vary in different cell types and during different time points of development. For example, *GABRA1* expression is very low in pre and perinatal rodent brain, but it increases dramatically after birth in all brain regions (Lopez-Tellez *et al.*, 2004). Similarly in humans, *GABRA1* expression was reported to gradually increases over the first postnatal years and is highly expressed during adulthood (Duncan *et al.*, 2010). In the adult mammalian brain, α1 is the most abundant $GABA_A$ receptor subunit and has been found to contribute to multiple different receptor subtypes (Pinto *et al.*, 2010).

One of the well-studied neurological disorders that has been associated with mutations in the *GABRA1* gene is epilepsy. While not all cases of epilepsy have a known genetic cause, to date, researchers have identified over 30 mutations in *GABRA1* that have been linked to various types of epilepsy (Steudle *et al.*, 2020). Altered expression of GABA related genes including *GABRA1* have also been reported in the prefrontal cortices and superior temporal gyrus of schizophrenia patients (Frajman *et al.*, 2020).

While most drugs that are currently being used to treat epilepsy and schizophrenia do not specifically target the α1 subunit, but the $GABA_A$ receptor is a whole, recent pharmacological studies have found that zolpidem, a $GABA_A$ receptor