REVIEW ARTICLE

Immunohistochemistry as a detection tool for ion channels involved in dental pain signaling

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Abstract *Background:* Despite advances in pain detection, diagnosis, and management, the prevalence of dental pain is still on the rise. Although dental pain is not directly related to fatal outcomes, the two most common types of dental pain—dental caries and dentin hypersensitivity—have a significant impact on an individual's quality of life. Understanding the mechanism of the pain pathway is one of the crucial steps in providing better treatment for these patients. Ion channels are critical biomolecules that have been the subject of dental study owing to their roles in the transmission and transduction of external stimuli, as well as in the control and perception of pain. Numerous immunohistochemical (IHC) staining approaches have also been used to identify the many ion channels implicated in peripheral pain signaling in dental pulp.

Highlight: This review highlights the critical steps in IHC and its role in the detection of ion channels involved in the dental pain signaling pathway.

Conclusion: The key ion channels identified using IHC and whose functions have been widely researched in dental tissues are addressed in this review article.

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1. Introduction

Dental pain is a common complaint seen daily in dental clinics. It refers to pain that arises from tooth or dental structures—the most common are dental caries and dentin hypersensitivity (DH). Despite the advanced methods available for dental pain detection, diagnosis, and management, the prevalence of these two pain-related dental diseases is still on the rise. A systematic review of the Global Burden of Diseases (GBD) reported that oral conditions contributed to a 20.8% increase in disability-adjusted life years (DALYs) from 1990 to 2010; the most prevalent oral condition being untreated dental caries in permanent teeth (Marcenes et al., 2013). A subsequent review of GBD on oral conditions in 2015 showed only a slight improvement in oral health conditions in low-income countries (Kassebaum et al., 2017). Studies have also shown that DH affects at least 10% of the global population, compromising their quality of life due to the discomfort and pain associated with DH (Exarchou et al., 2019, Gillam, 2021, Idon et al., 2019, Soares et al., 2021).

During dental caries progression, the demineralization of enamel and dentin layers enables bacterial invasion into the dental pulp, the innermost layer of the tooth structure rich in vasculatures and innervations. Consequently, a series of inflammatory reactions and an increase in intrapulpal pressure occur within the pulp chamber, which sensitizes the free nerve endings in the dentin-pulp complex and triggers the pain signaling pathway. The free nerve endings can also be triggered by external sources—thermal, chemical, and mechanical stimuli—which result in a condition known as DH. According to the most accepted theory of DH, these stimuli trigger dentinal tubule fluid movement, which in turn excites the free nerve endings at the dentin-pulp complex leading to DH (Braennstroem and Astroem, 1964).

Pre- and post-operative pain control plays a critical role in providing excellent dental management. Pharmacological management is the most common approach to managing dental pain, with its novel approaches are continuously being introduced. Current pharmacological management usually involves several types of analgesics, such as acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), and opioids, depending on the severity of pain. Advances in research and technologies have provided improved and additional treatment options for dental pain. Research is underway to find new targets for pharmacological approaches, as well as the use of biomaterials, stem cells, exosomes, and tissue regeneration to manage dental pain (Schuh et al., 2019).

Numerous ion channels have been implicated in peripheral pain signaling, including dental pulp. Temperature-sensitive, mechanosensitive, ligand-, and voltage-gated ion channels have received much attention due to their roles in mediating peripheral pain signaling. These ion channels have been thoroughly investigated in the search for new molecular targets for pain management. In particular, the investigation into transient receptor potential (TRP), the ion channel family responsible for thermo- and mechano-sensitive transduction, has led to the development of TRP channel-related analgesia, providing new insights for new drug designs in pain management (Bamps et al., 2021). However, its clinical use in humans is still questionable, as its application in animals and early

Phase II clinical trials has produced unacceptable adverse effects related to changes in body core temperature (Bamps et al., 2021).

In pursuit of better options for successful dental pain management, new molecular targets have been continuously investigated. Various investigation tools are available to achieve different research objectives, such as immunohistochemistry (IHC) and western blot for the detection of protein expression, polymerase chain reaction (PCR) and gene analysis for the detection of gene expression, and electrophysiology to explore and determine the functional aspects of target proteins and genes. In this regard, studying the expression of target proteins in dental tissues, especially dental pulp, is important, as the level of expression could serve as an indicator that may reflect its function therein (Kang et al., 2015, Nazıroğlu and Braidy, 2017, Zakir et al., 2020). The improvement of the tools currently available is ongoing, with the invention of novel methods also keeping pace. This review highlights the critical steps in IHC, emphasizing its use in the detection of ion channel proteins involved in dental pain signaling.

2. Materials and methods

Using the keywords 'dental pulp', 'immunochemistry', 'dental pain', and 'ion channel', our literature search performed via PubMed and Google Scholar from 2001 to 2021 found 14 publications reporting IHC investigations into ion channels in dental pulp associated with dental pain signaling. The IHC of these ion channels is discussed in Section 4.

3. Immunohistochemistry (IHC)

A laboratory technique involving the formation of antigen–antibody complex and its labeling via various methods, IHC has developed throughout the past century (Childs, 2014). Today, different methods are available for different types of specimens and for cellular focus (Mori and Cardiff, 2016). The term IHC describes the combination of immunological reactions (immuno-) performed in biological cells (-histo-), which occur via chemical reactions (-chemistry). It enables the detection of antigens in target tissues in both healthy and diseased conditions. This technique adopts the principle of natural body response, whereby antibodies are produced as part of the immune response in the presence of foreign antigens. Despite the availability of more advanced and state-of-the-art techniques, Brandtzaeg (1998) stated that IHC still plays an important role in designing further experiments, serving as one of the foundations of biomedical research.

3.1. History of IHC techniques

The ideation of IHC began in the 1890s with the production of anti-toxin (or better known today as antibody) for diphtheria bacteria, discovered by Emil von Behring (Behring and Kitasato, 1890). This finding became a breakthrough discovery that significantly benefited patients suffering from diphtheria and tetanus during that era. The IHC protocol was pioneered by Paul Erlich, who teamed up with Behring to understand more about antigen–antibody complexes. Early work on IHC staining by Erlich used aniline dye to categorize the blood cells and the staining method for tubercle bacilli, which is the principle for Gram stain today (Buchwalow et al., 2015). Advances in IHC progressed further with the work of John R. Marrack, who pioneered the labeling of antigen–antibody complex by attaching the antityphoid and anticholera antibodies with a red dye formed by tetrazotized benzidine (Marrack, 1934). The labeling of the antigen–antibody complex was further improved by Albert H. Coons, who used a fluorescent dye—the apple green color fluorescein isocyanate—that gives a brilliant greenish-yellow glow in the dark images (Coons et al., 1941). Fluorescein was extensively used as a fluorescent probe in immunolabeling. However, the development of cyanine and Alexa Flour dyes, which are more stable and available in more variations of excitation and emission wavelengths, has made fluorescein less commonly used today.

Despite the superiority of the immunofluorescence labeling technique, it cannot be imaged under an electron microscope (EM), a tool that detects ultrastructural images at the subcellular level. This problem has led to the idea of tagging antibodies with enzymes, pioneered by Graham and Karnovsky (1966).

Two common enzymes used as tags are alkaline phosphatase (AP) and horseradish peroxidase (HRP), which act upon colored substrates such as 3,3′-diaminobenzidine tetrahydrochloride (DAB). Under an electron microscope, DAB precipitates indicate the ultrastructural colocalization of the antigen–antibody complex (Graham and Karnovsky, 1966). Peroxidase-antiperoxidase (PAP) (tagged by the HRP enzyme) (Crowe and Yue, 2019), alkaline phosphatase-antialkaline phosphatase (APAAP) (tagged by the AP enzyme) (Di Cataldo et al., 2012), and avidin–biotin complex (ABC) methods (Hsu et al., 1981) were popular methods used for immunolabeling. As the endogenous peroxidase enzyme is preserved after paraffin embedding and cryopreservation (freezing), quenching of the endogenous peroxidase enzyme is imperative in the peroxidase detection method to prevent false-positive staining. Today, a polymer-based method (Sabattini et al., 1998) is gaining more interest among researchers, as the use of a large number of enzymes conjugated with secondary antibodies on the polymer increases the sensitivity of this detection method while circumventing biotin non-specific binding in the ABC method.

3.2. Principles of antigen–antibody complex and IHC staining

3.2.1. Types of antibodies for IHC

Most antibodies developed for IHC are bivalent Immunoglobulin G (IgG) molecules. The basic structure of IgG consists of two identical light chains and heavy chains held together by disulphide and noncovalent bonds, which give the whole antibody structure a schematic Y shape. Immunolabeling can be performed using either direct or indirect immunolabeling techniques. Direct immunolabeling requires only the presence of a primary antibody (Fig. 1A), which is attached to a specific site on an antigen termed an epitope. This technique requires an abundance of primary antibodies, which are often costly and not readily available.

In the indirect immunolabeling technique, a secondary antibody binds to the preformed primary antibody-antigen complex. In this case, the label (fluorophores or enzymes) is attached to the secondary antibody. Hence, the indirect
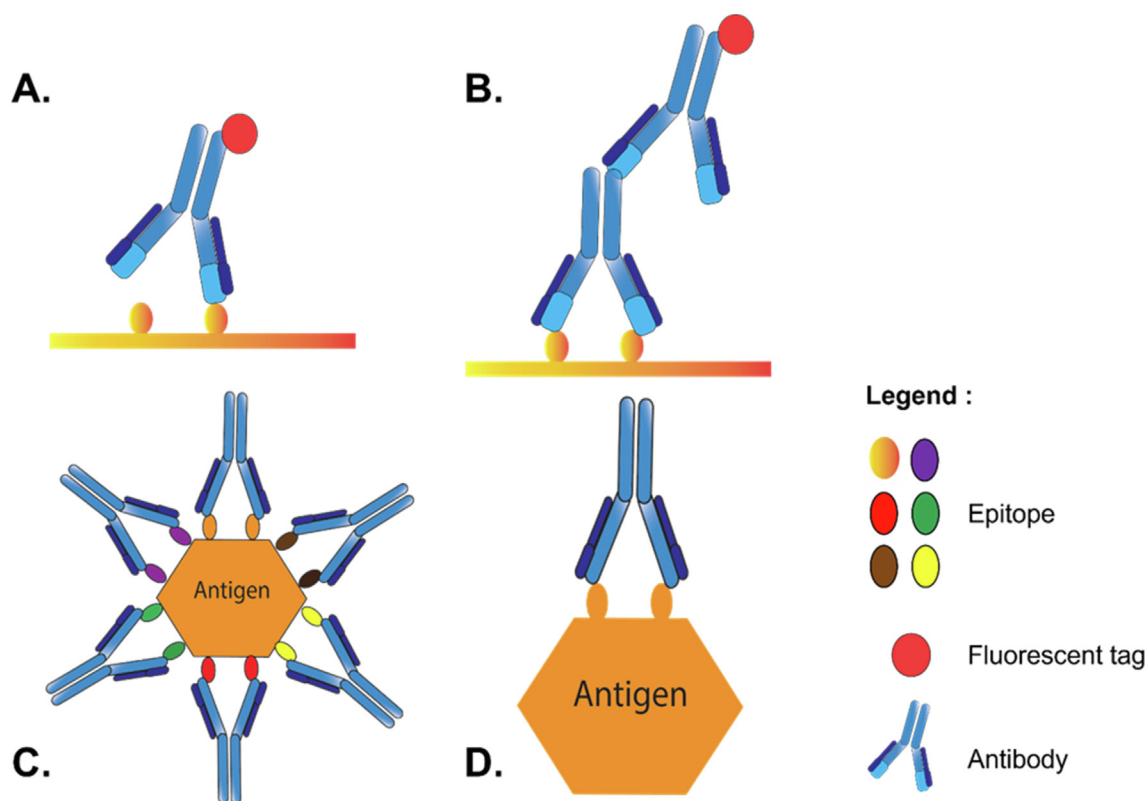
Fig. 1 Schematics of principles of immunolabelling. (A) Direct Immunolabelling. Primary antibody provides the antigenic-binding site that binds to it corresponding epitope, (B) Indirect immunolabelling. This technique involves a primary antibody that binds to its epitope and a secondary antibody that binds to the primary antibody, (C) Polyclonal antibody. Each antibody recognises different epitopes on a given antigen, (D) Monoclonal antibody. Monoclonal antibody recognizes only one epitope on an antigen.

immunolabeling technique does not require the abundance of primary antibodies (Fig. 1B).

The two types of antibodies available for IHC are monoclonal and polyclonal antibodies. Polyclonal antibodies are more widely used in biomedical research due to their lower cost and comparable sensitivity to monoclonal antibodies. Polyclonal antibodies are produced by introducing a large number of different B lymphocytes that recognize different epitopes on an antigen, which later differentiate into plasma cells and antibodies. Different antibodies that recognize different epitopes on the same antigen are produced; hence, the name is polyclonal (Fig. 1C).

Although cross-reactivity may occur with polyclonal antibodies, as they may recognize an epitope on a non-target antigen, they have a lower affinity to other antigens than that they are produced against. Indeed, the sensitivity of polyclonal antibodies has been shown to be comparable to that of monoclonal antibodies (Ascoli and Aggeler, 2018, Smedley et al., 2007, Varma et al., 2002), which are produced from one type of B lymphocyte and thus recognize only one epitope on an antigen (Fig. 1D). Therefore, monoclonal antibodies are highly specific but may be less sensitive than polyclonal antibodies. In recent years, the production of monoclonal antibodies has improved using DNA recombinant technology. Known as recombinant monoclonal antibodies, they are developed via a known nucleic acid sequence and cloning and consequently do not require the use of animals for their production (Kunert and Reinhart, 2016). The differences and benefits of using each polyclonal and monoclonal antibody are summarized in Table 1.

3.3. Parameters for successful IHC staining

Indeed, IHC plays a crucial role in research laboratories and serves as a guide for subsequent experiment planning. Experiment planning and data interpretation are equally important in determining the reliability of the data produced. To quote Mighell, Hume, and Robinson in their critical review of IHC, ''Used appropriately, IHC is a powerful and useful research tool. Used inappropriately, IHC produces confusing and misleading information'' (Mighell et al., 1998). In this regard, several guidelines have been outlined to guide researchers in producing robust and convincing data from their IHC experiments (Alexander et al., 2018, Bishop et al., 2018, Janardhan et al., 2018).

3.3.1. Tools and methodology for IHC staining

Prior to performing IHC experiments, the selection of tissue samples was a crucial step that influenced the outcome of the experiment. When animal samples are used, the experiment must comply with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines to ensure that adequate details regarding the use of animals are reported, enabling the readers to evaluate the methodology and reproducibility of the findings (Percie du Sert et al., 2020). This guideline

Table 1 Monoclonal and Polyclonal antibodies: differences and benefits (Ascoli and Aggeler, 2018, Lipman et al., 2005).

Character	Monoclonal	Polyclonal
Epitope	Single-epitope binding properties (homogenous)	Multi-epitope binding properties (heterogenous)
Binding specificity	Highly specific, thus a small change in the structure of epitope e.g. genetic polymorphism, glycosylation and denaturation may affect monoclonal antibody binding	Less specific; may recognise non-target antigen with similar epitope (cross reactivity)
Binding sensitivity	Less sensitive	More sensitive
Production	Production takes longer time and expanses. Once produced, the resource is constant and renewable	Can be generated more rapidly at lower cost, however antibody generated in multiple animals may differ among immunised animals
Biophysical diversity	Lack of biophysical diversity • highly susceptible to environmental changes such as pH and salt concentration, • may require stabilizing agent at low concentration to prevent precipitation	Its biophysical diversity allows for; • better stability when environmentally challenged; more stable over broad pH and salt concentration, • easier storage and dilution

was first introduced in 2010, following discussion and consensus by an international working group, and has since been updated according to progress in animal use in research. Other critical steps in IHC assays include tissue fixation and determination of the sensitivity and specificity of primary antibodies.

3.3.1.1. Tissue fixation. Tissue fixation allows the preservation of tissue morphology, prevents degradation of cell components, particularly antigens and enzymes, preserves antigen immunoreactivity, and facilitates antibody penetration during immunostaining procedures (Berod et al., 1981, Hayat, 2002). The most common fixative agent used is 10% formalin, with the duration of fixation varying depending on the type and size of the tissue. As minor irreversible alterations of the protein structure may occur during formalin fixation (Schacht and Kern, 2015), these antigens can be 'retrieved' to allow optimum antibody recognition by boiling tissue sections in hot water or using buffer solutions such as ethylenediamine tetra-acetic acid (EDTA) and citrate buffer (Shi et al., 2011).

3.3.1.2. Antibody specificity and sensitivity. The selection of antibodies for IHC is the key to reliable and successful experiments. The use of some antibodies may have been established in laboratory research, and therefore, their specificity and sensitivity are well characterized, in which cases the citation of the previously established protocol is mandatory. However, some antibodies are still new, and reports on their application for IHC are lacking. Thus, researchers are obliged to validate the specificity and sensitivity of their antibodies.

Antibody sensitivity refers to the amount of antibody required to produce positive staining. A highly sensitive primary antibody requires only a small amount of antibody to detect the target protein and can be used at high dilutions and vice versa; a poorly sensitive antibody requires a large amount of antibody for protein detection and must be used at low dilutions. For an unknown or new antibody, its sensitivity is determined via optimization, performed using several different dilutions of that new antibody, from low to high dilutions. Other factors that are critical during optimization are the choice of buffer used for antibody dilution, the temperature during the experiment, and the incubation period of the primary antibody (Janardhan et al., 2018, Walker, 2006). However, a sensitive antibody may not necessarily be able to

detect the target protein. The ability of an antibody to detect a target protein is termed specificity, assessed by the inclusion of additional experiment groups, using the same protocol used for the primary antibody. These are the positive and negative control groups that are mandatory for validating the observations and findings from IHC experiments. Details of these control groups have been explained in several IHC guidelines (Alexander et al., 2018, Hewitt et al., 2014).

3.3.2. Image analysis and data interpretation

The interpretation of IHC data has evolved over the past few decades. From the qualitative and subjective assessments of the presence or absence of immunostainings, today the data is interpreted more objectively via various quantification methods. Semi-quantitative and computer-assisted quantification has been developed and is widely used today (Di Cataldo et al., 2012, Fassler et al., 2020, Kaczmarek et al., 2004, Matos et al., 2010, Taylor and Levenson, 2006, Walker, 2006). This technique defines positive staining by setting the intensity threshold for positive pixels and negative pixels, as well as weak and strong positive pixels, and has improved the reproducibility of IHC data (Chlipala et al., 2020).

3.3.2.1. Semi-quantitative method. In an attempt to improve IHC data interpretation, several methods have been developed using semi-quantitative techniques. In 1988, Gundersen proposed a method based on point counting to estimate the volume, surface area, and length of labeled structures (Gundersen et al., 1988). In 2001, Klein proposed a scoring method to determine one additional parameter for image analysis—the staining intensity—which he used to determine the prognosis of thyroid cancer (Klein et al., 2001). Although the measurement of staining intensity is useful in interpreting IHC data, the intensity score examined and assessed by experienced researchers or clinical specialists takes longer, is prone to human bias, and is more subjective and therefore less reliable (Seidal et al., 2001). These disadvantages of the semi-quantitative method were improved by the development of a computer-assisted method, with the data interpretation comparable to that of semi-quantitative methods but with an objective assessment of staining intensity, avoiding scoring disagreement between researchers (Cross, 2001, Matos et al., 2006).

3.3.2.2. Computer-assisted quantification method. The computer-assisted quantification method has gained much interest in biomedical research due to its convenience, reproducibility, and reliability for IHC data interpretation (Di Cataldo et al., 2012). This method, also known as computer-assisted imaging (CAI), uses algorithms to identify a labeled structure, usually by physical differences, such as color, to produce the required measurement outcomes—volume, surface area, length, and staining intensity of the labeled structures. Today, different CAI algorithms are available to analyze images from different tissues and IHC staining techniques for both biomedical research and diagnostic laboratories (Dixon et al., 2015, Fassler et al., 2020, Guirado et al., 2018, Jawad and Abdullah, 2017, Lee et al., 2019, Tewary et al., 2021).

Using image analysis software and plugins to analyze large data more consistently, CAI requires minimal work performed by researchers. Introduced more than two decades ago (Collins, 2007), ImageJ was the first free open-source software to provide image analysis tools and is widely used for biological image analysis. More recently, free open-source software, such as CellProfiler and L-measure, has also been available.

ImageJ owes its strength to its numerous plugins and its ability to support various file formats. However, to some degree, the high number of plugins leads to overlapping functions and usability. A more recent image analysis software program, CellProfiler, has gained much interest among cell biologists. Introduced in 2006 (Carpenter et al., 2006), CellProfiler has similar and comparable advantages to ImageJ, except for the smaller number of plugins available. A review of 15 free available software programs for biological image analysis ranked CellProfiler first in terms of usability and functionality (Wiesmann et al., 2015).

The analysis of the complex neuron morphology, which consists of dendrites, cell bodies, and axons, is best performed using L-measure software. The algorithm of this freely available software has the ability to extract large data for neuronal image analysis. L-measure software considers the length, bifurcation, and number of branches of each dendrite of a neuron, making it a suitable tool for morphometric measurement (Scorcioni et al., 2008).

Despite being frequently cited in the literature, most studies that applied the aforementioned software programs provided only minimal information regarding the protocol for image analysis, resulting in difficulty in data reproducibility. In this regard, Crowe and Yue (2019) produced a comprehensive step-by-step protocol for image analysis using ImageJ Fiji software, with no specific plugin applied. Used to analyze the expression of organic anion transporting polypeptide (OATP1B1) stained with DAB and hematoxylin in genotyped human liver tissue, this protocol has been successfully adopted by more recent research published in renowned journals (Chu and Yeh, 2020, Tajan et al., 2021, Wu et al., 2021).

4. The involvement of ion channels in dental pain signaling

4.1. The general sensory system

The sensory system comprises the central nervous system (CNS) and the peripheral nervous system (PNS). The main structures that constitute the CNS are the spinal cord and the brain. Peripherally, the primary afferent fibers whose cell bodies reside in the dorsal root ganglia (DRG), or the trigeminal ganglion (TG) for afferent fibers originating from the orofacial region, form the PNS. Pain signaling begins when pain receptors or nociceptors on the free nerve endings are triggered by noxious stimuli, such as extreme temperatures and inflammatory mediators, released during inflammation. The free nerve endings of the primary afferent fibers are found on the skin, internal organs, joints, muscles, and dental pulp. Upon stimulation, the painful stimulus is transduced into electrical impulses at the free nerve endings, followed by transmission of electrical impulses via the first, second, and third order neurons that ascend to the brain where pain is perceived. The perception of pain is not a straightforward process as pain transmission can be modulated by various factors at the level of the spinal cord and the brain. Studies on these structures, including the expression of biomolecules that may be involved in dental pain signaling, are ongoing. The results from these studies will shed light on understanding the mechanism of pain and, more importantly, on finding molecular targets that can alleviate dental pain.

4.2. IHC for detecting ion channels involved in dental pain signaling

Within a tooth structure, any stimulus that triggers the free nerve endings present within the dentin-pulp junction produces pain similar to that found in other tissues. However, due to some peculiarities in neuronal fiber phenotypes in dental pulp (Fried et al., 2011, Gibbs et al., 2011, Paik et al., 2009) and the types of stimuli that trigger dental pain (as defined by the term DH), the general mechanism of pain may not be exclusively inferable to dental pain. For this reason, research focusing on dental pain mechanisms has been conducted to explore the potential molecular targets that may play a significant role in dental pain signaling.

Numerous ion channels in the PNS interact with each other within the dental pain signaling pathway. Ion channels are one of the important biomolecule components that have gained much interest in the dental field due to their roles in the transduction and transmission of external stimuli, as well as modulation and perception of pain. Ion channels are specialized proteins in the plasma membrane that serve as a passage for charged ions to cross the plasma membrane, following their electrochemical gradients. The movement of these ions initiates and activates various downstream signaling pathways, including the pain pathway. In dental pulp, several ion channels involved in peripheral pain signaling have also been discovered. They are TRPV1, TRPV4, TRPA1, and TRPM8, ATP receptors and purinergic receptor (P2X3), pannexin 1-ATP permeable channel, sodium channel family (Nav1.7, Nav1.8, and Nav1.9), and voltage-gated calcium channels (Cav1.2 and Cav3.1) (Alavi et al., 2001, Bakri et al., 2018, Beneng et al., 2010, El Karim et al., 2011, Ju et al., 2015, Kim et al., 2020, Liu et al., 2015, Michot et al., 2018, Park et al., 2006, Renton et al., 2003, Renton et al., 2005, Sampoerno et al., 2020, Wells et al., 2007, Westenbroek et al., 2004). Here, we highlight the antibody-antigen complex detection method, control groups, and methods for data analysis related to these ion channels. A summary of the IHC studies on these ion channels is also presented in Table 2.

Table 2 Immunohistochemical detection of ion channels involved in dental pain signalling.

No.	Ion channel (s)	Immunolabelling technique	Data interpretation technique	Sample	Reference
1.	VR1 (TRPV1) and P2X3	Enzymatic labelling intensified using nickel ammonium sulphate-intensified diaminobenzidine. Positive and negative controls included, negative control performed by replacing primary antibody with normal rabbit serum or omission of primary antibody. Antibody specificity test included.	Imaging tool: video linked to a microscope. Image analysis: computer-assisted image analysis with details of determination of immunoreactive fibers included.	Human tooth pulp	(Renton et al., 2003)
2.	TRPV1, TRPA1 and TRPM8	Immunofluorescence labelling. Negative control performed by omission of primary antibody	Imaging tool: Visual assessment, protocol not specified	Sprague Dawley Rat dental primary afferent neurons	(Park et al., 2006)
3.	TRPV1, TRPA1 and TRPM8	Enzymatic labelling (avidin–biotin peroxidase) for ex vivo dental pulp staining, immunofluorescence labelling for dental pulp culture.	Imaging tool: Confocal microscope (fluorescent images), light microscope (non-fluorescent images) Image analysis: protocol not included	Human dental pulp	(El Karim et al., 2011)
4.	TRPA1 and TRPM8	Immunofluorescence labelling. Negative control performed by omission of primary antibody.	Imaging tool: fluorescent inverted microscope Image analysis: NIH ImageJ analysis software. Details of determination of immunoreactive cells and analysis were included.	TG innervating dental pulp of C57B16 and TRPM8 knock-out mice, TRPM8-GFP-tagged) mice	(Michot et al., 2018)
5.	TRPV4	Hematoxylin and eosin, immunofluorescence labelling	Imaging tool: camera attached to a BX51 fluorescence microscope (fluorescent images). Image analysis: NIH ImageJ analysis software. Co-localization of primary antibodies were analyzed using Co-localization Color-Map plugin in ImageJ software. Details of determination of immunoreactive fibers and analysis were included.	Human dental pulp, healthy and pulpitis	(Bakri et al., 2018)
6.	ATP receptors (P2X3)	Enzymatic fluorescence labelling. Negative control performed by omission of primary antibody or replacement of primary antibody with non-specific rabbit IgG.	Imaging tool: visual assessment Image analysis: immunoreactive fibers translated into percentage	Human dental pulp	(Alavi et al., 2001)
7.	Tuj-1 (neural stem cell marker) Panexin 1-ATP permeable channel	Immunofluorescence labelling	Imaging tool: visual assessment Image analysis: protocol not specified	Human dental pulp	(Liu et al., 2015)
8.	Nav1.8	Enzymatic labelling (avidin–biotin peroxidase). Positive and negative controls included, negative control performed by replacing primary antibody with normal rabbit serum or omission of primary antibody. Antibody specificity test included.	Imaging tool: video linked to a microscope. Image analysis: computer-assisted image analysis with details of determination of immunoreactive fibers included.	Human dental pulp	(Renton et al., 2005)
9.	Nav1.9	Immunofluorescence labelling	Imaging tool: confocal laser scanning microscope Image analysis: ImageJ analysis software. Details of determination of immunoreactive cells and image analysis were included.	Human dental pulp and TG	(Wells et al., 2007)
10.	Nav1.7	Enzymatic labelling (avidin–biotin peroxidase)	Imaging tool: Scoring method of staining intensity assessed via visual	Human dental pulp	(Beneng et al., 2010)

Table 2 (continued)

No.	Ion channel (s)	Immunolabelling technique	Data interpretation technique	Sample	Reference
			observation by two independent blinded observers Image analysis: protocol not included		
11.	Nav1.7	Hematoxylin Mayer	Imaging tool: visual assessment Image analysis: protocol not included	Sprague Dawley rats dental pulp	(Sampoerno et al., 2020)
12.	Cav1.2 (L-type calcium channel)	Enzymatic labelling (gold and DAB-pre-embedded). Negative control performed by omission of primary antibody or via pre-incubation with peptide against which primary antibody was raised.	Imaging tool: electron microscope Image analysis: quantification performed by counting gold particles in odontoblast and fibroblast that contained nucleus.	Sprague Dawley Rat dental pulp	(Westenbroek et al., 2004)
13.	Cav1.2 (L-type calcium channel)	Immunofluorescence labelling	Imaging tool: Visual assessment of fluorescent images performed using fluorescent microscope Image analysis: protocol not included	Dental pulp stem cells	(Ju et al., 2015)
14.	Et alCav3.1 (T-type calcium channel)	Immunofluorescence labelling. Negative control performed by omission of primary antibody. Protocol conformed with ARRIVE guidelines for preclinical studies.	Imaging tool: Confocal microscope Image analysis: images were processed with Zen software (Carl Zeiss) - protocol not specified	Rat odontoblast cell culture, dental pulp tissue	(Kim et al., 2020)

4.2.1. Antibody-antigen complex detection method

Depending on the study protocol, especially the type of tissue used, different studies have adopted different techniques for immunohistochemical labeling to enable the best visualization of their target ion channels. The investigation of the expression of ion channels in human dental pulp was mostly performed via enzymatic labeling (Alavi et al., 2001, Beneng et al., 2010, El Karim et al., 2011, Renton et al., 2003, Renton et al., 2005, Westenbroek et al., 2004) to enable ultrastructural labeling within the tissue, as described in the previous section. Two studies used hematoxylin and eosin to compare the control group (healthy pulp tissue) with pulpitis (Bakri et al., 2018, Sampoerno et al., 2020), while immunofluorescent labeling was mostly used to label ion channels in experiments involving cell cultures (Ju et al., 2015, Kim et al., 2020, Michot et al., 2018).

4.2.2. Control groups

In line with the guidelines for submission by Alexander et al. (2018), most studies conformed to these recommendations to ensure their data reproducibility. All authors have reported the use of negative controls for their experiments by omitting primary antibodies or by replacing primary antibodies with normal serum. Studies that compared asymptomatic and symptomatic dental pulp or treated and non-treated groups used asymptomatic and non-treated groups, respectively, as controls. However, only two studies included positive controls in their papers (Renton et al., 2003, Renton et al., 2005). As many researchers have overlooked the importance of including positive controls in their experiments, the need to report positive controls must be further emphasized to confirm that true-positive staining is observed in the tissue of interest.

4.2.3. Data analysis

Data analysis is a crucial step in IHC to ensure data reproducibility. Thus, it is important to include and explain the protocol used to analyze IHC images, such as the determination of positive staining, selection of sample and area for analysis, as well as the software and plugins used. Surprisingly, only six out of 14 publications provided a detailed protocol for IHC data analysis. Except for a study by Kim et al. (2020), other studies that did not include a detailed protocol performed their data analysis via visual assessment. However, it is interesting to note that one study reported the use of two independent blinded observers to analyze IHC images (Beneng et al., 2010). This is indeed a good example of performing data analysis; using more than one assessor who is blinded reduces the bias that may result in single and unblinded observation.

Sharing research findings that benefit the scientific community is noble, provided that the correct research method is used and accordingly reported in publications. The methodological considerations required for publication to ensure data reliability and reproducibility, as outlined by Alexander et al. (2018), are summarized in Table 3.

5. Future direction of IHC in dental pain signaling

Biological and physiological activities, such as pain signaling, are governed by the presence of numerous amino acids and proteins within a biological system. Thus, studying the expression of these biomolecule components in both healthy and diseased conditions has made a major contribution toward understanding normal physiology and the changes that lead to pathology. Amino acid and protein detection via IHC is a simple and convenient method that has led to major discoveries in the biomedical sciences. However, these discoveries could

Table 3 Methodological considerations for data reproducibility in IHC staining (Adapted from Alexander et al., 2018).

Methodology	Experimental and analytical considerations
Staining	1. Description of primary and secondary antibodies (source, species the antibody was raised, epitope it was raised against, and the diluting buffer) 2. Blocking methodology 3. Sample preparation – isolation, extraction and storage duration and temperature 4. Positive and negative controls

Imaging	1. Autofluorescence from proteins or compounds which are intrinsically fluorescent may contribute to false-positive immunoreactivity. Common sources of autofluorescence are:
	  a. Cellular structures – mitochondria, lysosomes
	  b. Intercellular structures – collagen, elastin
	  c. By-product of fixative process – Schiff bases formation in extended aldehyde fixation
	
	2. Bleed-through – may occur if immunofluorescence staining is performed using two or more fluorescent labels. Using fluorophores with distinguishable/different emission and excitation profile would minimise bleed-through.
	3. Exposure time – longer exposure time increases possibility of autofluorescence and bleed-through. Images may oversaturate and the fluorophores fade.

Image analysis	1. A detailed protocol of image analysis must be included, for both manual scoring or computer-assisted quantification and image analysis. 2. Blind analysis must be performed, whether by different personnel or by blinding the sample labelled.
Image presentation for publication	Images of both lower and higher magnification should be presented to enable assessment of any unintentional or inappropriate image manipulation

only be beneficial and meaningful if the data produced were reliable and reproducible. Each step involved in IHC has its own role in achieving these objectives and is critical in producing robust and convincing data worthy of publication.

In the study of dental pain signaling, the detection of biomolecules via immunochemistry not only allows scientists to understand the mechanism of pain, but more importantly, it may shed light on new discoveries for the therapeutic management of pain and pave the way to a better quality of life. In addition, with the advance of fluorescence microscopy, IHC has recently been applied to imaging techniques using CLARITY and light sheet microscopy (França et al., 2019). This technique enables imaging of the rich vasculatures and primary afferents within the intact dental pulp in 3-dimensional (3D), serving as an outstanding tool to study the molecular and anatomical details in the whole dental tissue that aids in understanding dental diseases and their treatment.

6. Conclusion

With a long history of providing evidence for scientific discoveries in biomedical research, the application of IHC continues to serve this purpose today as a laboratory tool that works in parallel with more recent experimental techniques. As explained in this review, it is crucial to adhere to the current guidelines when performing the critical steps in IHC to ensure reliable and reproducible data. In the search to identify the biomolecules critically involved in dental pain signaling, data from IHC have provided anatomical and physiological insights that will guide scientists to move forward within this research niche.

Ethical statement

This narrative review was conducted ethically in all aspects. It is clear from any kind of plagiarism; all reviewed relevant articles and resources were properly cited. As this is a narrative review which highlights the use of IHC in dental pain research, it did not require approval from the Institutional Ethical Committee.

CRediT authorship contribution statement

Rosmaliza Ramli: Writing – original draft, Visualization. **Siti Norasikin Mohd Nafi:** Writing – review & editing. **Nor Azura Ahmad Tarmidzi:** Writing – review & editing. **Nurulezah Hasbullah:** Writing – review & editing. **Nurhafizah Ghani:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sdentj.2022.02.004.

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