PURINERGIC SIGNALING IN PAINFUL PULPITIS

Lily Pachanin See

A DISSERTATION

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Degree of Doctor of Science in Dentistry
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Purinergic Signaling in Painful Pulpitis

THESIS

Presented to the Faculty of Penn Dental Medicine in Fulfillment of the Requirements for the Degree of Doctor of Science in Dentistry

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ACKNOWLEDGEMENTS

It takes a village to raise a child, and likewise it takes a village to get one through a doctoral degree. With that said, I’d like to take a moment to acknowledge and thank my village without whom this journey would have been impossible. First and foremost, I thank my mentor, Dr. Claire H. Mitchell, for her patience, guidance, flexibility, and humor throughout these five years. From her, I’ve learned several valuable lessons whether it be the importance of controls, never forgetting a noun and verb in my Powerpoint titles, and how to think and troubleshoot like a scientist. I have the utmost gratitude for her mentorship and kinship.

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My deepest gratitude goes to my parents and brother for their unconditional love and support. They gave me the privilege to study abroad without worry, checked-in almost daily, and have supported all my decisions (whether they agreed with it or not!). Lastly, E’Tee, thanks for being my person.

To everyone that has been a part of my experience here at Penn, Thank you (x3).
Injury of the tooth pulp is often excruciatingly painful and yet the receptors and neural circuit mechanisms that transmit this form of pain remain poorly defined in both clinical human and preclinical rodent models. The role of purinergic pathways are of particular interest and potential relevance in dental inflammation and pain. This study examined the involvement of purinergic receptors in human dental inflammatory pain by comparing their distribution in healthy pulps compared to symptomatic and asymptomatic inflamed pulps, and then testing for sex differences in expression. Ionotropic P2X2R and P2X3R were selected for their implication in pain; whereas ecto-nucleotidase NTPDase1 (CD39) reflects extracellular ATP concentration. We found P2X3R and P2X2R, as well as CD39, to colocalize with protein gene product 9.5 (PGP9.5)-positive nerves in control tissues. P2X3R was additionally found on odontoblasts, and CD39 on other non-neuronal structures. Both immunohistochemistry and immunoblots demonstrated that all three proteins were significantly increased in symptomatic pulpitis, suggesting both receptors and agonist ATP were elevated with increased pain. Increased expression of P2X3 and CD39 were more frequently observed in women than men. Our findings support a role for increased purinergic signaling in humans with inflammatory dental pain, with preference in women. This differential response suggests purinergic signaling may contribute to sex differences in pain. To further study signaling pathways of painful pulpitis in the preclinical model, quantifiable behavioral models, namely the Mouse Grimace Scale (MGS) and Facial von
Frey assay, were evaluated for their validity in assessing dental pulp pain in mice. We found that unilateral pulp exposure injury to the first maxillary molar in mice resulted in significant increase in MGS from 24 h onward, indicating presence of spontaneous pain. Mechanical allodynia, assessed by the Facial von Frey assay, progressed more gradually with significant increases on both sides of the face on day 4, and unwillingness to tolerate filaments above 0.6 g by day 6 post-injury. This work demonstrates that we have clear, easily identifiable behavioral readouts for trigeminal nociception in the mouse following dental injury.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AIP</td>
<td>asymptomatic irreversible pulpitis</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene related peptide</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger associated molecular pattern</td>
</tr>
<tr>
<td>DPI</td>
<td>dental pulp injury</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>ENT</td>
<td>equilibrative nucleoside transporter</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma amino butyric acid</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>HTM</td>
<td>high threshold mechanoreceptor</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional animal care and use committee</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional review board</td>
</tr>
<tr>
<td>LTM</td>
<td>low threshold mechanoreceptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MGS</td>
<td>mouse grimace scale</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate (glutamate receptor)</td>
</tr>
<tr>
<td>NTPDase</td>
<td>ecto-nucleoside triphosphate diphosphohydrolase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>protein gene product 9.5</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PNI</td>
<td>peripheral nerve injury</td>
</tr>
<tr>
<td>SIP</td>
<td>symptomatic irreversible pulpitis</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>TG</td>
<td>trigeminal ganglia</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TMD</td>
<td>temporomandibular disorder</td>
</tr>
<tr>
<td>TRPA1</td>
<td>transient receptor potential ankyrin 1</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VFH</td>
<td>von Frey hair filaments</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 An overview of purinergic signaling

Purines, such as adenosine 5’-triphosphate (ATP), have well-established roles as intracellular energy currency with involvement in cell metabolism and replication. In the 1970s, Geoffrey Burnstock first proposed ATP to additionally function as a principle neurotransmitter with either release from non-adrenergic, non-cholinergic nerves or release as a co-transmitter with noradrenaline, acetylcholine, or other substances [32-35]. Since then, these purines have been found to be involved in various physiological and pathological processes including inflammation [73; 81], pain [36; 39], bone development and regeneration [203], circulation [81; 94], sleep and arousal [109; 164], and more. Their actions are mediated through purinergic receptors, which consist of different receptor families for adenosine (P1 receptors) and for ATP and ADP (P2 receptors) [1; 68]. Interestingly, ATP and adenosine regulate several physiological processes through counteraction of the other’s induced effects. Thus their respective levels require a balance that is tightly regulated through degradation of ATP by ectonucleotidases and subsequent degradation of AMP by ecto-5’ nucleotidase (CD73) leading to the generation of adenosine.

1.1.1 P2X receptors

ATP and ADP activate P2 receptors. Based on pharmacology, P2 receptors can be further subdivided into P2X and P2Y families [40]. P2X receptors are membrane ion channels formed as multimers of several subunits, each subunit composed of two transmembrane domains, an extracellular domain, and intracellular termini. There are seven known P2X receptor subunits to date: P2X1-P2X7. These receptors have been found to form either homomeric or heteromeric
channels, the different channels varying in their sensitivity to agonist/antagonist, channel kinetics, and function. Upon binding of extracellular ATP, these channels become permeable to cations, including Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\). This results in depolarization, opening of voltage-gated Ca\(^{2+}\) channels, and increased cell excitation [157]. For comprehensive reviews on P2X receptors see [20; 128; 249]. P2X receptors have received much attention for their involvement in various pain states [20], with P2X3R the most widely studied regarding nociception.

1.1.2 P2X3R and P2X2/3R

P2X3R are trimeric nonselective cation channels that are activated by extracellular ATP and its analogues. Each full length P2X3 subunit has a total molecular weight of 44.3 kDa or 50-75 kDa after N-glycosylation, due to four different glycosylation sites [76]. The distinctive characteristics of this ion channel include its high affinity desensitization (ATP EC\(_{50}\)=1 μM), fast desensitization onset (millisecond range), slow recovery from desensitization (minute range), and ability for heteromerization with P2X2 to generate non-desensitizing responses [98]. P2X3R is located on small to medium sized nerve fibers, predominantly C and A\(\delta\) fibers, suggesting specificity for nociception [38; 259].

In 1997, Cook et al. retrogradely labelled rat dental pulp afferents and demonstrated these nociceptive neurons to exhibit specific P2X3R immunoreactivity and a pharmacological profile that was not evident in non-nociceptive neurons (muscle-stretch receptors) [62]. Several studies ensued confirming prominent expression of P2X3R in animal sensory ganglia (30-60%): dorsal root ganglia (DRG), trigeminal ganglia (TG), and nodose ganglia [27; 176; 243; 259]. P2X receptors on sensory and sympathetic ganglion neurons are almost exclusively comprised of P2X2 and P2X3 subunits, as neurons from P2X2/P2X3\(^{Dbl^{-/}}\) mice showed minimal to no response.
to ATP [59]. Further investigations through pharmacological antagonism and knock-out mice demonstrated phenotypes with reduced pain-related behaviors, urinary bladder reflexes, airway hyperreactivity, and itch [60; 90; 238]. Due to its relative specificity for pain, P2X3R poses an attractive target in pain therapeutics with lower likelihood of adverse effects in the brain, gastrointestinal, or cardiovascular tissues, which remains a limiting factor for many existing pain therapeutics.

**Table 1-1 P2X3R vs. P2X2/3R**

<table>
<thead>
<tr>
<th></th>
<th>P2X3R</th>
<th>P2X2/3R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Desensitization (complete in)</strong></td>
<td>Fast (&lt;1s)</td>
<td>Slow (&gt;20s)</td>
</tr>
<tr>
<td>ATP (EC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>1 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>Large pore</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Agonists</td>
<td>ATP, αβ-meATP, ATPγS, 2-MeS-ATP, BzATP and Ap(n)A</td>
<td>Same as P2X3R homomer</td>
</tr>
<tr>
<td>Antagonists</td>
<td>(Non-selective) Suramin, PPADS, Aurintricarboxylic acid (Nucleotide-derived) TNP-ATP, 2',3'-O-Benzylidene-ATP, DT-0111 (Non-nucleotide derived) A317491, Gefapixant (AF-219), MK-7264, AF-353 (RO-4), AF-906 (RO-51), Selective for P2X3 homomers: IP51, AZ004, RO-85, mAb12D4, spinorphin, Eliapixant (BAY 1817080), BLU-5937, MK-3901, S-600918, HS10383</td>
<td>Same as P2X3R homomer</td>
</tr>
<tr>
<td>Major cellular expression</td>
<td>Primary sensory neurons</td>
<td>Same as P2X3R homomer</td>
</tr>
<tr>
<td>Major role</td>
<td>Pain, bladder reflex, cough</td>
<td>Same as P2X3R homomer with addition of taste</td>
</tr>
</tbody>
</table>

*This table is based on information from [20; 128; 242]

**Underlined antagonists are/have undergone clinical trials.**
**Figure 1-1 P2X3R vs P2X2/3R.** The P2X3 receptor can exist in both homomeric and/or heteromeric forms. These forms differ in channel kinetics and pore size. Activation of these receptors result in formation of nonselective cation channels. As the P2X2/3R displays sustained non-desensitizing responses, larger influx of cations are suspected with the heteromeric form. (Made in Biorender)

P2X3R exists in both homomeric and/or heteromeric forms (P2X2/3R) (**Figure 1-1**). Their characteristics are summarized in **Table 1-1**. Both forms present with high affinity desensitization, localization on small nociceptive neurons, and activation and inhibition by similar agonists and antagonists [20]. The two forms, however, differ in their channel kinetics and pore size. Homomeric P2X3R displays fast desensitization with slow recovery, where heteromeric P2X2/3R displays sustained non-desensitizing responses [20; 98]. The different kinetics may suggest that homomeric P2X3R contributes to acute pain, whereas heteromeric P2X2/3R modulates longer lasting sensitivity such as nerve injury or chronic pain. However, in vivo [149] and human [208] P2X3R have shown faster resensitization compared to their in vitro and rodent counterpart. This resensitization is also expected to occur faster in inflammation where there are rises in local tissue temperature [149], extracellular acidification [96], neuropeptides [66; 84] and cytokines [91] that facilitate recovery from desensitization. Further differences in P2X3 subtype distribution between human and rodents have been described. Notably, the P2X3 homomer subtype is the predominant P2X subtype in human DRG sensory neurons [229]; whereas P2X2/3 heteromers are higher in afferents of viscera [90]. This may suggest different therapeutic approaches to treat visceral or somatic pain in humans. Although
the precise functional differences between the two forms remains elusive, these receptors are well-recognized for their involvement in nociception with several clinical trials testing the therapeutic potential of available antagonists. Current and completed clinical trials regarding P2X3R are summarized in Table 1-2.

Table 1-2. P2X3R antagonists in clinical trials

<table>
<thead>
<tr>
<th>Drug (Antagonist)</th>
<th>Phase</th>
<th>Conditions</th>
<th>Completed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefapixant (AF-219/MK-7264)</td>
<td>2</td>
<td>Chronic cough, Idiopathic pulmonary fibrosis, Chronic cough, Idiopathic pulmonary fibrosis</td>
<td>Yes [2; 183]</td>
</tr>
<tr>
<td>Eliapixant (BAY 1817080)</td>
<td>2</td>
<td>Endometriosis (pain), Chronic cough, Endometriosis (pain), Overactive bladder, Diabetic neuropathic pain, Chronic cough</td>
<td>Yes [89]</td>
</tr>
<tr>
<td>HS-10383</td>
<td>1</td>
<td>Chronic cough</td>
<td>No</td>
</tr>
<tr>
<td>S-600918</td>
<td>2</td>
<td>Chronic cough</td>
<td>Yes</td>
</tr>
<tr>
<td>BLU-5937</td>
<td>2</td>
<td>Chronic cough, Chronic pruritis, Atopic dermatitis</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*This table is based on information from clinicaltrials.gov

**If available, publications based on results of clinical trial are referenced.

1.1.3 Ectonucleotidases

Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) are a family of ectoenzymes that hydrolyze extracellular ATP and ADP, modulating the activity of these purines on P2 receptors. The cascade of dephosphorylation started by NTPDases is continued by CD73 with the hydrolysis of AMP to adenosine. Lastly, adenosine levels are regulated by ecto-5′-nucleotidase (ADA) and adenosine deaminase (EC 3.5.4.4) converting adenosine to inosine (Figure 1-2).
Figure 1-2 ATP hydrolysis cascade. Through a cascade of dephosphorylation, ATP is hydrolyzed to ADP or AMP, and further into adenosine. These purine metabolites activate different purinergic receptors, resulting in different downstream signaling pathways. Thus, the action of ectonucleotidases is of critical importance in regulating these signaling pathways (Made in Biorender).

There are eight different NTPDase members. NTPDases 1, 2, 3, and 8 are membrane-bound enzymes with an extracellular facing catalytic site. NTPDases 5, 6 are located intracellularly and are found in either a membrane-bound or soluble secreted state. NTPDases 4, 7 are located entirely intracellularly, on Golgi membranes and lysosomal vacuoles [21], and intracellular membranes [232]; respectively. The membrane-bound NTPDases (1, 2, 3, 8) are the ectonucleotidases that control availability of extracellular nucleotide agonists against P2 receptors.

NTPDase 1, 2, 3, 8 are firmly anchored to the membrane by two hydrophobic N-terminal and C-terminal transmembrane domains with an extensive extracellular loop. NTPDase1-3 form homo-oligomeric assemblies and have been found as dimers to tetramers. Unlike P2X receptors, hetero-oligomeric complexes of NTPDases have not been reported to date. Oligomerization of NTPDase reveals increased catalytic activity and the state of oligomerization can affect catalytic properties [226; 244; 262]. The different membrane-bound NTPDases can be differentiated based on substrate preference, divalent cation usage, product formation, and catalytic properties.
NTPDases are expressed in virtually all mammalian tissues, but especially in vasculature, immune cells, and neuronal cells.

| Table 1-3 Comparison of membrane-bound NTPDases |
|------------------------------|----------------|----------------|----------------|----------------|
| Substrate preference         | NTPDase1       | NTPDase2       | NTPDase3       | NTPDase8       |
| Divalent cation usage        | ATP ~ ADP      | ATP >>> ADP    | ATP > ADP      | ATP > ADP      |
| Optimal pH for maximal activity | 7 - 9.5       | 4.5 – 8.5     | 4.5 - 11       | 4.5 – 8.5      |
| ATP K_m (human, µM)          | 17             | 70             | 7              | 46±5 (mouse, µM) |
| ADP K_m (human, µM)          | 22             | ND             | 23             | 265±20 (mouse, µM) |

*This table was adapted from [159] with addition of information from [214].
**ND: not determined as ADP are poor substrates of NTPDase2

1.1.4 NTPDase1 (CD39)

NTPDase1, or CD39, is the best characterized NTPDase with high efficacy of limiting purinergic activity through direct conversion of ATP to AMP due to high affinity for both its substrates [159]. The full structure of CD39 revealed that ATP and ADP are hydrolyzed at the same site, sequentially, demonstrating the effective removal of ATP by CD39 [273]. Each CD39 oligomer has a molecular mass of 70-100 kDa with six potential glycosylation sites [145]. CD39 has been reported to preferentially exist as assemblies from dimers to tetramers, with oligomerization increasing catalytic activity [140; 226; 244; 262].

As the rate-limiting enzyme in the ATP/ADP-AMP-Adenosine pathway, CD39 has become recognized as a checkpoint for anti-inflammatory immune response [9; 250], as ATP has largely pro-inflammatory action whereas adenosine has largely anti-inflammatory action. Interestingly, expression of CD39 has additionally been shown to change linearly with levels of
extracellular ATP over a physiologically relevant range in epithelial cells [179] and astrocytes [178] suggesting CD39 may also serve as a marker for increased levels of extracellular ATP. This was not found with other membrane-bound NTPDases, NTPDase2 or NTPDase3 [179].

1.1.5 P1 receptors

Through the cascade of ATP breakdown, ATP is eventually hydrolyzed to adenosine which further acts upon P1 or adenosine receptors. P1 receptors are G protein-coupled receptors (GPCR) with four subtypes identified to date: ADORA-1, ADORA-2A, ADORA-2B, ADORA3. The ADORA-1, 2A, and ADORA-3 receptors possess high affinity for adenosine (EC$_{50}$ = 0.01-1 μM) while the ADORA-2B demonstrates lower affinity for the substrate (EC$_{50}$ = 24 μM) [92; 116]. This suggests ADORA1, 2A and ADORA-3 capable of responding to adenosine in physiological concentrations, whereas ADORA-2B is only activated in pathological conditions. ADORA-1 and ADORA-3 receptors couple to G$_i$ proteins resulting in inhibition of adenylate cyclase and cAMP generation, and increased activity of phospholipase C (PLC). In contrast, ADORA-2A and ADORA-2B receptors couple to G$_s$ proteins resulting in increased intracellular cAMP accumulation, activation of protein kinase A (PKA), and phosphorylation of cyclic AMP response element binding protein (CREB). All four subtypes can couple to mitogen-activated protein kinase (MAPK), giving them a role in cell growth, survival, death, and differentiation.

P1 receptors are widely expressed throughout the body: along the nervous, cardiovascular, respiratory, and immune system [206]. Thus, these receptors regulate various physiological functions including sleep, cognition, cardiac function, nociception, and wound healing [136]. For comprehensive reviews on P1 receptors see [25; 136; 231]. In regards to
nociception, the ADORA-1 receptor is the most characterized regarding its role in anti-
ociception and pain modulation.

1.1.6 ADORA-1

ADORA-1 predominantly plays a role in the nervous system, with its highest levels
found in the brain especially at excitatory nerve endings [69]. Other than inhibition of adenylate
cyclase, at the neuronal level ADORA-1 additionally stimulates K⁺ channels while inhibiting P-
Q- and N-type Ca²⁺ channels [25], resulting in a reduction of neuronal signaling and
hyperpolarization of neurons. The receptor additionally modulates the release of
neurotransmitters, such as glutamate, GABA, acetylcholine, and serotonin, at synaptic regions
[64].

ADORA-1 has been found to reduce inflammatory and neuropathic pain [239; 240; 279],
specifically reducing mechanical allodynia and thermal hyperalgesia [118; 222], with the anti-
ociceptive effects of acupuncture attributed to ADORA-1 activation [278]. ADORA-1 knockout
mice demonstrate increases in hyperalgesia, anxiety, hypoxic damage, lipolysis, insulin
secretion, osteopetrosis, and susceptibility to epilepsy [141]. Similarly, with its broad systemic
effects, pharmacological studies have shown that full ADORA-1 agonists have serious side
effects [150], which make them impractical for clinical use. However, partial agonists have
shown some promise with anti-hyperalgesic effects and reduced side effects [224; 257].

1.2 ATP and adenosine in inflammation and pain

ATP and adenosine have been well documented for their opposing actions in
inflammation and pain [26; 83; 132; 222; 278]. In inflammation, ATP is recognized as a mostly
pro-inflammatory molecule; whereas adenosine has been recognized for its mainly anti-inflammatory effects [26; 83]. Similarly, in pain, ATP is recognized for its role in pain initiation and sensitization [132]; whereas adenosine has garnered attention for its potent antinociceptive effects [278]. These opposing effects are a result of their action on different receptor families. With an understanding of the receptor families and purinergic pathway, this section will cover the actions of ATP and adenosine in inflammation and pain.

1.2.1 Purines in inflammation

Extracellular ATP is a recognized danger associated molecular pattern (DAMP) and triggers pro-inflammatory action through activation of P2 receptors [135]. In contrast, adenosine is a potent immunosuppressor, acting through P1 receptors [116]. The balance between extracellular ATP and adenosine is critical to immune homeostasis, emphasizing the importance of CD39 or NTPDase1, the rate-limiting enzyme in ATP hydrolysis [275].

In physiological conditions, there are low extracellular levels of both ATP (400-1000 nM) and adenosine (40-80 nM) [83]. This contrasts to the high intracellular levels for ATP (3-10 mM) [102]. ATP is used for intercellular communication, by neuronal cells as a neurotransmitter and by non-neuronal cells for local signaling [37; 88]. It is released extracellularly by various mechanisms in response to mechanical deformation, hypoxia, and various agents [24; 37]. This includes passive release through ruptured cell membranes and active release by activated or stressed cells through vesicular exocytosis, or transport via membrane bound channels or transporters (Figure 1-3) [77]. ATP release is commonly mediated by immune, neuronal, and endothelial cells [83]. Extracellular adenosine levels largely depend on the hydrolysis of
extracellular ATP with some endogenous release via equilibrative nucleoside transporters (ENTs).

In inflammation, ATP is released from cells leading to three fold or more increase in extracellular ATP concentration [24]. ATP activation of various P2 receptors results in chemotaxis of inflammatory cells [50; 158], production of oxygen free radicals by neutrophils [12], and production of cytokines by inflammatory cells [134]. ATP-mediated inflammation is largely attributed to activation of P2X7R and the NLRP3 inflammasome complex [49]. Interestingly, ATP can also have anti-inflammatory effects when present in lower micromolar levels with chronic exposure [72; 115].

Following the inflammatory process, there are increases in adenosine levels due to hydrolysis of ATP by CD39. Adenosine activation of P1 receptors results in inhibition of endothelial cell adhesion [117], reduction in superoxide anion production by neutrophils [15], reduction of inflammatory cytokines [114], facilitation of anti-inflammatory cytokine release [156], and production of vascular endothelial growth factor (VEGF) [113; 228]. Adenosine-mediated anti-inflammation is largely mediated by ADORA-2 receptors [87; 167].
**Figure 1-3 ATP release.** ATP is released extracellularly by passive release through ruptured membranes or active release through activated or stressed cells. This released ATP further activates purinergic receptors, and its action is limited by hydrolysis through ectonucleotidases. (Made in Biorender)

1.2.2 Purines in pain

Initial reports linking ATP to pain originated from studies injecting ATP into human blisters and eliciting a pain response [22; 23]. ATP induced a dosage-dependent pain sensation that increased in intensity and duration at higher concentrations [22; 110]. Hamilton et al. demonstrated that ATP ionophoresis in humans induced a burning pain sensation, depended on capsaicin-sensitive neurons, and was augmented in the presence of inflammatory mediators [110]. Following the cloning and characterization of purinergic receptors, Burnstock put out a unifying purinergic hypothesis for the initiation of pain in conditions such as sympathetic pain, vascular pain, and cancer pain [36]. Since then, it has become recognized that ATP is mechanistically involved in pain induction through both neuronal and nonneuronal cells, acting at peripheral and central sites. Specifically, ATP induces pain activation in peripheral sensory nerve fibers [38], mediates cross-talk between primary and secondary nociceptive neurons as well as with astrocytes and microglia [130; 131], and has functional interactions with transient receptor potential V1 (TRPV1) and glutamate (NMDA) receptors [215; 252].

ATP induces pain activation in peripheral sensory nerve fibers primarily through P2X3R. P2X3R is selectively expressed on small non-peptidergic nociceptive fibers [38], found along the entire sensory pathway (neuronal cell bodies, free nerve endings, as well as central axonal terminals) [259], upregulated and sensitized in inflammation [268], and has been suggested to be involved in inflammatory and neuropathic pain. ATP directly acts on P2X3R on peripheral nerve endings, inducing depolarization of the nerve and activation of pain. Centrally, activation of P2X3R of the central terminal increases glutamatergic signaling, where α,β-meATP increases
excitatory postsynaptic currents (EPSC) frequency [169; 193; 194]. P2X3R additionally has functional interactions with TRPV1 receptors. Studies have shown that TRPV1 and P2X3 have different interactions in vivo under different pathological conditions [85; 154; 220; 271]. For example, in trigeminal inflammatory pain, using the temporomandibular disorder (TMD) model, P2X3R and TRPV1 demonstrated mutual facilitatory interaction [220]; whereas in DRG neuropathic pain, using the spared nerve injury model, P2X3R facilitated TRPV1 while TRPV1 inhibited P2X3R [271]. Further details on the P2X3 receptor have been discussed in Section 1.1.2 of this dissertation. ATP is additionally found to induce neuropathic pain through P2X4, P2X7, and P2Y receptors on microglial cells [222]. For reviews of P2X4, P2X7, and P2Y in neuropathic pain see [20; 130; 222].

Adenosine has been found to regulate pain transmission at the spinal, supraspinal, and peripheral level [223]. Intrathecal and intravenous injected adenosine in human volunteers reduced experimentally induced allodynia [222]. ADORA-1 in particular produces prominent antinociception at both the peripheral and spinal level [180; 222]. Peripherally, ADORA-1 reduces cyclic AMP production, Ca2+ entry, and decreases the release of calcitonin gene related peptide (CGRP) [44; 107]. Centrally, ADORA-1 increases K+ conductance and hyperpolarization of dorsal horn neurons and inhibits peptide and glutamate release [4; 162; 236]. Further details on the ADORA-1 receptor have been discussed in Section 1.1.6 of this dissertation. Less is known regarding the ADORA-2A, 2B, and 3 receptors in pain. The role of these receptors in pain is still a matter of debate, as both pro- and anti-nociceptive effects have been reported [180; 223].
1.3 Pulp biology overview

The dental organ is believed to receive purely nociceptive innervation [6; 123], and has thus been commonly used as a model to study nociceptors and peripheral pain mechanisms. However, due to the dental pulp’s unique situation within a rigid encasement, there are several distinctive constraints on its development, maintenance, and response to injury. Depending on the status of the tooth, a stimulus can produce acute/nociceptive dental pain, dentin sensitivity, or inflammatory dental pain. Towards an understanding of those differences, the following two sections will cover an overview of pulp biology, and the progression and characteristics of dental inflammation and dental pain.

1.3.1 Dental pulp anatomy

The tooth is primarily composed of three components—enamel, dentin, and pulp. On a weight basis, enamel is the most mineralized hard structure of the human body (at 96%), dentin (70%) and bone (~65%) [101]. Enamel forms an insulating barrier that functions to protect the tooth from physical, chemical, and thermal stimuli that would otherwise be injurious to the underlying structures [160]. This structure lacks any self-renewal capacity. Dentin is formed immediately subjacent to enamel. Dentin is a tubular structure composed of minerals and organic material, and dentinal fluid. Unlike enamel, dentin is continuously formed throughout life both physiologically, and in response to stimulation [101]. This leads to high variability in the sizes of pulp chambers between different and even within the same individual. In the core of the tooth resides the dental pulp. The dental pulp is primarily a connective tissue composed of fibroblasts, collagen fibers, undifferentiated mesenchymal stem cells, and vascular, lymphatic, and nervous
elements. Pain transmission is attributed to the dental pulp nerves (central pulp) and pulpo-dentin complex (peripheral pulp).

**Table 1-4 Classification of dental nerves**

<table>
<thead>
<tr>
<th>Myelination</th>
<th>Fiber</th>
<th>Size</th>
<th>Conduction speed</th>
<th>Excitability threshold</th>
<th>Response</th>
<th>Possible function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelinated</td>
<td>Aβ</td>
<td>0.5-12 μm</td>
<td>30-70 m/s</td>
<td>&lt; 9.9 uA</td>
<td>Well-localized, sharp</td>
<td>Pre-pain, (pain, dentinal sensitivity?)</td>
</tr>
<tr>
<td></td>
<td>Aδ</td>
<td>0.2-5 μm</td>
<td>12-30 m/s **</td>
<td>Low 9.9 uA</td>
<td>Well-localized, sharp</td>
<td>Pain, dentinal sensitivity</td>
</tr>
<tr>
<td>Unmyelinated</td>
<td>C</td>
<td>0.4-1.2 μm</td>
<td>0.5-2 m/s</td>
<td>High 37.4 uA</td>
<td>Diffuse, dull, burning, referred pain</td>
<td>Delayed pain, neurogenic inflammation</td>
</tr>
</tbody>
</table>

*adapted from [17; 18; 112], mostly based on electrophysiological recordings from major nerve trunks after dentin stimulation in experimental animals
** Aδ fast fibers; *** Aδ slow fibers

The dental pulp is densely innervated by primary sensory neurons (~90%), comprising of C, Aδ, and Aβ fibers from the trigeminal ganglion. Apart from sensory innervation, the pulp additionally receives sympathetic fibers from the superior cervical ganglion (~10%) [41; 42].

Classical studies have demonstrated that, at the root entrance at the main apical foramen, nerve bundles innervating the pulp are mostly unmyelinated C-fibers (75-80%), and the majority of the myelinated fibers are Aδ-fibers (~90%) [41; 124; 125; 192]. The differences between these nerve fiber types are summarized in **Table 1-4.** Throughout the core of the pulp, these nerve bundles travel as fascicles near blood vessels with little branching. In the crown or coronal region, there is extensive branching, known as the Plexus of Rashkow, with myelinated nerve fibers losing their myelin-sheath in this zone and terminating as free nerve endings. These free nerve endings can be found extending in the cell-rich zone, cell-free zone, odontoblast layer, and even up to 0.2 mm into dentinal tubules [104; 192]. A summary and diagram of the peripheral pulp can be found in **Table 1-5,** and **Figure 1-4.**
### Table 1-5 Peripheral pulp compartments

<table>
<thead>
<tr>
<th>Peripheral pulp compartment</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plexus of Rashkow</td>
<td>Large vessels, nerve fiber plexus (myelinated/unmyelinated fibers), fibroblasts, perivascular cells, undifferentiated mesenchymal cells</td>
</tr>
<tr>
<td>Cell-rich zone</td>
<td>Fibroblasts, perivascular cells, undifferentiated mesenchymal cells, capillaries, unmyelinated nerve fibers, dendritic cells, defense cells</td>
</tr>
<tr>
<td>Cell-free zone (zone of Weil, subodontoblast layer)</td>
<td>Capillaries, unmyelinated nerve fibers, collagen (von Korff) fibers, processes of fibroblasts (no cell bodies)</td>
</tr>
<tr>
<td>Odontoblast layer</td>
<td>Odontoblasts, unmyelinated nerve fibers, terminal capillaries, dendritic cells, von Korff fibers</td>
</tr>
<tr>
<td>Dentin</td>
<td>Odontoblastic process (up to 4 mm), free nerve ending (up to 0.2 mm), dentinal fluid</td>
</tr>
</tbody>
</table>

![Figure 1-4 Pulpo-dentin complex](image)

**Figure 1-4 Pulpo-dentin complex.** A diagram representative of the different layers of the pulpo-dentin complex, where (A) dentin, (B) odontoblast layer, (C) cell free zone, (D) cell rich zone are displayed (Made in Biorender).

### 1.3.2 Dental pulp afferents

The Aδ and C fibers are the key nociceptive fibers of the dental pulp. They have been classified as (1) specific high threshold nociceptive fibers that provide warning of tissue damage with preference to a stimuli type (ie. mechanical, thermal), and (2) polymodal receptors with a wide dynamic range, responding to multiple types of stimuli, that have various regulatory functions including vasoregulation, inflammation, and facilitation of immune and healing responses through the release of neuropeptides [42]. The Aβ fibers are considered low threshold
mechanoreceptors (LTM) with suspected roles in prepain [186] and occlusal mechanosensation [75]. The Aδ fibers can be further divided into two groups: Aδ fast fibers and Aδ slow fibers [16; 270]. The majority of Aδ fibers are Aδ fast fibers or Type 1 high-threshold mechanoreceptors (HTM). Most of these fibers innervate the peripheral pulp (ie. odontoblast layer, inner dentin) in the coronal region. These fibers respond to both mechanical and chemical stimuli but have relatively high heat thresholds. The remainder of Aδ fibers are Type 2 Aδ slow fibers. These fibers have lower heat threshold but high mechanical threshold and are primarily found in the pulp core. They are insensitive to hydrodynamic stimuli and are typically silent nociceptors, only activated by intense heat or cold [270]. C fibers are polymodal receptors with high thresholds for activation. Like the Aδ slow fibers, they are typically silent nociceptors and more likely to activate by intense temperature changes or chemical stimuli such as inflammatory mediators. Interestingly, C fibers are less susceptible to hypoxia and does not require as much oxygen as Aδ fibers to function due to their smaller size [201].

Although the nerves innervating the pulp are primarily of small to medium diameter, findings in experimental animals have suggested that pulp afferents represent pre-terminal portions originating from larger myelinated fibers at more proximal locations [123]. This has been corroborated by observations that individual pulp axons exhibit higher conduction velocities at extrapulpal than intrapulpal segments [43; 173], the inferior alveolar nerve has low proportions of C-fibers [142], retrograde tracer experiments label mostly large and medium sized neurons [245; 246], and some unmyelinated nerve fibers in human pulp express markers for myelinated fibers [119]. This suggests that the proportion of C, Aδ, and Aβ fibers may be different than previously described.
1.3.3 Pulpo-dentin complex and hydrodynamic theory

Although dentin and pulp have different structures and compositions, they are functionally coupled and continuous in their reaction to stimuli. Due to dentin’s tubular nature, it is a permeable structure and allows movement of its fluid contents to activate mechanosensitive structures in dentin and pulp. This phenomenon was termed the hydrodynamic theory and first proposed by Gysi in 1900 [105]. It was further supported by the works of Brännström and colleagues [29]. When dentin is exposed, it is subject to considerable chemical, mechanical, thermal, and microbial stimulation. Depending on the magnitude of resulting fluid shifts, odontoblasts and pulpal nerve fibers can be directly activated. This leads to pulpal responses where pulpal cells may produce dentin, nerves, and/or blood vessels. As nerve endings terminate in inner dentin, much focus has been directed to odontoblasts whose processes extend much deeper into dentin and thus may be the first to detect stimuli.

Odontoblasts are complex cells with roles as (1) a natural barrier between dentin and pulp, (2) in regulation of dentinogenesis, (3) in antimicrobial defense and immune function, and (4) in dentinal sensitivity, though much controversy has surrounded this suspected function. Odontoblasts lack direct connections to nerve fibers, whether through synaptic or gap junctions, which have suggested that they lack sensory function [41]. However, various ion channels (ie. TRP, Piezo, voltage-gated Na+ channels) have been demonstrated on odontoblasts indicating a capability to generate receptor potential [237]. Recently, paracrine communications between odontoblasts and neighboring cells by ATP has been identified [174]. Through activation of TRP or Piezo channels, among other channels, odontoblasts release ATP further activating purinergic channels on neighboring odontoblasts, nerve fibers, and/or blood vessels [79; 200]. Although odontoblasts cannot independently transduce a nociceptive signal, the concept of odontoblasts as
a supporting cell, interacting with nerve fibers to regulate environmental conditions, is gaining traction.

Hydrodynamic forces on odontoblasts and nerve fibers are attributed to nociceptive pain responses and dentinal sensitivity. In health, normal pulp tissue pressure is around 10 mmHg [17]. As pulp tissue pressure is higher than the oral cavity, there is a constant gentle outward flow of fluid in dentinal tubules [121]. This is beneficial to the dental pulp in preventing diffusion of toxins towards the pulp. In the intact tooth, the enamel layer acts as a buffer dissipating the effects of stimuli and minimizing pulpo-dentin reactions. Intense thermal stimuli are required to elicit reactions, or changes in dentinal fluid flow. For example, when intense cold thermal stimuli such as refrigerant spray is applied to the intact tooth surface, an increased outward flow of dentinal fluid causes sharp pain that quickly dissipates with removal of the stimuli [184]. This is associated with activation of Aδ fast fibers. Alternatively, extreme heat causes increased inward flow and delayed dull pain, which was not attributed to activation of Aδ fast fibers [43; 185]. Intense heat stimuli activate C fibers or Aδ slow fibers at the pulp core that have lower heat thresholds. These are acute nociceptive responses providing warning signals prior to tissue damage. The mechanism of dentinal sensitivity similarly relies primarily on Aδ fast fibers. In dentinal sensitivity, dentinal tubules are exposed allowing direct tactile, thermal, osmotic, or evaporative stimuli to elicit fluid movements and sharp pain. Repeated stimulation can lead to sensitization, resulting in the sharp pain lingering for longer periods. Collectively, nociceptive pain, with the exception of intense heat stimuli, and dentinal sensitivity primarily rely on hydrodynamic forces in dentin eliciting a stimuli from Aδ fast fibers.
1.4 Pulpal inflammation

1.4.1 Pulpal inflammation and pain (symptomatic irreversible pulpitis)

Pulpal inflammation is a direct result of damage to tooth structure, usually of carious nature, eliciting inflammatory responses in the pulp. These inflammatory responses include immune responses, dentin formation, and changes to the vascular and neuronal networks [95]. For reviews of the immune and dentin responses, see [86; 95]. Pulpal inflammation is deemed irreversible once bacteria gains access to the pulp or if any evidence of necrosis is found within the pulp [213; 227]. Microcirculation and sensory nerve activity are considered the two key components of the pulp inflammatory process [151]. The main actions on microcirculation include alterations in pulpal blood flow and increases in capillary permeability; whereas changes to the neuronal network include activation, sensitization, and increases in nerve fibers [112].

Towards vascular responses, inflammatory mediators induce neurogenic inflammation causing vasodilation of arterioles and increased permeability of capillary beds. These reactions result in increased pulp fluid volume. Kim et al. demonstrated that the moderately inflamed pulp showed increases of pulpal blood flow over 40% [152]. Due to the dental pulp’s rigid encasement, this increased blood flow results in increases in pulp tissue pressure, where pulp tissue pressure can increase over three times its normal values [17; 253]. This leads to compression of the venular system and accumulation of metabolic product and mediators, further instigating inflammation and, if not resolved, eventually pulpal death [152]. Previously, strangulation theory was hypothesized, where it was believed that the entire pulp would swell and degenerate as a unit. However, this theory was quickly refuted as these changes were found to be localized to the initial site of injury and to circumferentially spread if not resolved [256]. This compartmentalization of injury is due to the protective mechanics of the pulp [112; 122].
the area of injury, increases in pulp pressure leads to reduced capillary filtration due to the hydrostatic pressure gradient. In nearby uninflamed tissue, there are increases in capillary absorption and increased lymph outflow to reduce both interstitial tissue fluid volume and protein concentrations. Overall, these protective mechanics help restrict increases in pressure to the site of injury [112; 122].

The neuronal network responds to inflammation by extensive anatomical and cytochemical changes, typically resulting in pain. The barrage of inflammatory mediators leads to axon reflexes, increased neuropeptide expression, remodeling of ion channels and receptors, nerve sprouting, and activation and/or sensitization of nociceptors (ie. reduced threshold for firing). Increased axon reflexes cause release of proinflammatory mediators from nerve endings, further instigating neurogenic inflammation [196; 202]. Thus, substance P (SP) and calcitonin gene related peptide (CGRP) increase as a gradient related to inflammation and pain levels [46-48; 100]. TRPV1 and tetrodotoxin (TTX)-resistant Na\textsubscript{v} channels are likewise increased in inflammation [58; 263; 265]. In the case of TTX-resistant Na\textsubscript{v} channels, this affects a clinician’s ability to achieve effective dental anesthesia. Normally silent nociceptors become activated [42; 196]. For example, C-fibers are activated by the increase in histamine and bradykinin available in inflammation. Inflammation further lowers the threshold of nociceptors where increases in systolic pressure can activate mechanosensitive neurons (resulting in throbbing pain) [42; 195], and normal physiological temperature can activate thermosensitive neurons (resulting in spontaneous pain) [56; 247]. The combined action of activated silent nociceptors, reduction in threshold, and nerve sprouting increases the overlap of receptive fields and enhances the spatial summation of nerve activity [153; 196]. This collectively increases pain intensity in response to dentin and pulpal stimulation. Cumulatively, these peripheral responses lead to peripheral
sensitization characterized by spontaneous pain, hyperalgesia, and allodynia. Central responses additionally affect the pain experience, including central sensitization and referred pain. For more information on these central aspects, see [112; 218; 270]. If not treated, these neuronal and vascular changes can continue as a vicious cycle until eventual pulp necrosis (Figure 1-5).

Figure 1-5 Pulp compartmentalization and vicious cycles of inflammation. A. Inflammatory changes to the pulp are localized to the site of injury and circumferentially spread if treatment is not administered. B. Vascular changes in inflammation leading to eventual pulp necrosis. Diagram modified and reprinted with permission from Dr. Syngecuk Kim [151]. C. Neuronal changes in inflammation that lead to peripheral sensitization and pain.

1.4.2 Asymptomatic irreversible pulpitis (painless pulpitis)

Interestingly, pulpitis can progress to pulp necrosis without any symptoms. The prevalence reported is of wide range (26-60%) [17; 189]. However, the mechanisms for asymptomatic irreversible pulpitis are still not well understood. In line with symptomatic pulpitis, there are suspected peripheral and central contributions. Peripheral nociceptors express receptors for opioid, cannabinoid, somatostatin, adrenergic subtypes, neuropeptide Y and gamma amino butyric acid (GABA) that inhibit nociceptor activity [78]. There may be local release of these inhibitory mediators activating GPCRs coupled to G\(_i\) proteins that inhibits PKS action and
reduces nociceptor function. Some studies have shown differences in inflammatory mediators between symptomatic and asymptomatic irreversible pulpitis samples [80; 187; 221], suggesting possible differences in inflammatory pathways. Local degeneration of pulp axons has additionally been suggested [270]. Centrally, there are suspected actions of interneuron modulation and descending inhibitory pathways. Inhibitory interneurons can release inhibitory neurotransmitters, such as GABA, decreasing nociceptive input of primary afferents to central projection neurons. In descending pathways, neurons in the periaqueductal grey, nucleus raphe magnus and dorsal raphe can also release inhibitory neurotransmitters from the descending axon terminals to the projection central neuron [191]. Alternatively, Khabbaz et al. studied the effects of bacterial byproducts on pain and inflammation. Their group found bacterial endotoxins in all samples with pulpitis, where symptomatic samples had greater endotoxins than asymptomatic samples [147; 148]. Collectively, both host-derived and bacterial-derived factors may affect the presence or lack of symptoms, however further study is needed for a conclusive understanding.

1.4.3 Behavioral assessment models for dental inflammatory pain

Animal models are widely used for dental research due to their availability and ethical concerns with human participants. Towards the dental injury model, the animal’s dental pulp is commonly exposed and left open, to simulate chronic inflammatory changes seen in the clinic [42]. Timing is an important feature to consider as pulpal, neural, and inflammatory conditions differ for acute injury to normal tissue compared to transient acute inflammation, prolonged acute inflammation, and chronic inflammation [61; 196; 251; 269]. Notably, chronic inflammation presents with invasion of immunocompetent cells that then affect pulpal and neural injury reactions [19; 144].
A recurring disadvantage with the animal model is that there have been little behavioral data to correlate specific findings with specific sensory conditions [42]. Generally, studies have considered the animal’s normal behavior, as it is a good index of relative well-being. This includes analyses of feeding behavior, weight gain, mobility, curiosity, and grooming in murine animals. However, these analyses are limited in their specificity and translational capacity. In the dental office, mechanical hypersensitivity and spontaneous pain are particularly problematic for patients and there are common behavioral assessment tools for these in rodent models when the pain occurs elsewhere in the body [82]. To date, only a handful of studies using the dental pulp injury model have examined animal behavior, and these studies have not incorporated these common assays used to measure nociception [97; 172; 230].

Towards mechanical pain measurement, the predominant assessment tool in rodents are reflexive withdrawal assays in which calibrated von Frey hair filaments (VFHs) are applied to the hind paw and the experimenter determines which filaments evoke paw withdrawal [28]. VFHs can also be applied to the face, and has been demonstrated to be capable of quantifying TMD pain [241]. However, it has yet to be applied for dental inflammatory pain. Facial von Frey is described in more detail in Chapter 2.

Towards spontaneous pain measurement, the Mouse Grimace Scale (MGS) interrogates facial expressions including the positioning of the mouse nose, cheek, ear, eye, and whiskers [161]. An advantage of MGS over reflexive assays is that spontaneous pain resembles pain reports in the clinic and facial expressions are used in the clinic to measure pain in infants, although these assays are currently not as high-throughput in rodents in delivering stimuli and recording immediate responses. Together however, both reflexive and spontaneous measurements of pain-like behavior provide advantages in that they can be performed without
anesthesia, invasive implants, or time intensive tasks performed by the animal that require long term learning and memory, which may mean that the interpretation of the behavior may be confounded by factors outside the animal’s pain level. Like the von Frey assay, MGS has not been used to examine dental inflammatory pain.

1.5 Purinergic signaling in dental pain

Purinergic receptors are preferentially expressed in TG nociceptive neurons compared with that in the DRG, which may confer dental orofacial pain with its unique properties [10; 11]. The tooth is a unique organ in that the only sensation transmitted is pain [6; 123]. This is mediated by dental nerve fibers and odontoblasts. Notably, purinergic involvement in pain was first confirmed by patch-clamping rat dental pulp afferents [62]. Several preceding studies found purinergic immunoreactivity in small diameter cell bodies in the DRG, which suggested involvement in pain [27; 176; 243; 259]. However, they were unable to conclude these neurons and purinergic activity were nociceptive, until the use of dental pulp afferents. This section will review the current knowledge of purinergic signaling in dental pain.

1.5.1 Purinergic distribution in dental health

Alavi et al. were the first to demonstrate purinergic signaling in the human dental pulp, showing positive immunoreactivity to P2X3R on nerve fibers and odontoblasts [8]. This study launched general interest of purinergic nociception within the dental pulp. Subsequently, Liu et al. examined the expression and function of cell surface located NTPDases in the human pulp [174; 175]. They found positive immunoreactivity of NTPDase 1, 2, 3 and did not report their findings of NTPDase8. NTPDase1 and 3 were found along blood vessels and the odontoblast
layer, and NTPDase1 was additionally found along the subodontoblast region. Contrarily, NTPDase2 was found along pulpal nerve fibers, colocalizing with Schwann cells [175]. Enzyme histochemistry revealed functional ATPase activity along the areas of NTPDase distribution [174]. Their group further confirmed the presence and function of ATP release receptors (ie. Pannexin-1, Pannexin-2, and connexin-43) in the healthy human pulp [174; 175]. Pannexin-2 and connexin-43 had immunoreactivity limited to the odontoblast layer. Pannexin-1 immunoreactivity was found on both the odontoblast layer and nerve fibers. These *ex vivo* human studies provided concrete evidence for the presence of purinergic machinery in the healthy pulp, suggesting a role for purinergic signaling in acute dental pain and sensitivity.

1.5.1 Purinergic signaling in acute nociception and dentin sensitivity

Further evidence for purinergic signaling in acute dental pain and sensitivity was demonstrated by ATP release from odontoblasts, and induction of central sensitization following activation of P2X3R positive dental pulp afferents. Using an *ex vivo* tooth perfusion model, cold and mechanical external dentin stimulation was found to cause ATP release. This could largely be blocked using Pannexin channel blockers [174]. Further studies attributed the ATP release to activation of various sensory receptors (ie. TRP channels, Piezo channels) on odontoblasts [79; 127; 197; 200; 233]. The increase in ATP was presumed to activate P2X3R on dental pulp nerve fibers, initiating a pain response. Subsequently, P2X3R functional activity in dental pulp afferents was examined using central sensitization models in murine animals. Direct application of P2X3R agonists to the fresh exposed murine dental pulp was found to increase central sensitization through increases in pERK expression, mechanoreceptor field size, and responses to noxious stimuli as measured from single neuronal activity [5; 52]. These changes could be
reversed through the application of P2X3R antagonists [5; 52; 126]. Collectively, these studies demonstrated a role for purinergic signaling in acute dental pain and sensitivity, through the activation of sensory receptors on odontoblasts to release ATP through Pannexin-1 channels and stimulate P2X3R-positive nerve fibers.

1.5.3 Purinergic signaling in dental inflammatory pain

Fewer studies have investigated the effect of purinergic signaling in dental inflammatory pain. This may be due to a lack of accessible validated behavioral models to assess orofacial pain. Chen et al. demonstrated that LPS-induced pulp inflammation in rats increased expression of P2X2R, P2X3R, and P2X5R in the trigeminal ganglion [51], however this study did not have a functional read-out for pain. Other *ex vivo* studies found upregulation of connexin-43, NLRP3, and caspase-1 expression with increasing severity of pulpitis [139; 171]. Likewise, these studies did not assess the patient’s level of pain associated with the dental pulpitis. Thus, these studies suggest increases in ATP availability and purinergic activity in pulpitis; however, their contributions to pain are unclear. With the addition of the behavioral models described in this dissertation, recent studies have implicated a role for P2X7R in painful pulpitis [129; 133; 267; 274]. Other than its actions in nociception, purinergic signaling has also been found to affect dental wound healing, tooth movement, and bone healing. For reviews on purinergic action in oral tissues see [170; 277].

Collectively, these studies have demonstrated a potential role for purinergic signaling in pulpal inflammation and pain. Purinergic machinery is present in the healthy human pulp, and increased in inflammatory disease in the murine model. It is unclear whether human tissue will reflect the same increases in inflammatory disease and its effect on clinical pain. Further research
in murine models additionally require establishment of murine pain assessment models. Herein, it is crucial to identify markers relevant to disease in human patients, and develop pain assessment models to allow further mechanistic studies in the murine model.

1.6 Dissertation aims

Dental pain, though physiologically present to serve a protective function, can become pathologic and debilitating if treatment is not rendered in a timely manner. Pulpitis is highly associated with significant spontaneous pain and pain triggered by cold foods and mastication. Endodontics has focused on the alleviation of that pain for the past several decades; however, it is not well-understood which receptors and signaling mechanisms confer the dental pulp with its unique pain qualities. Furthermore, the current available analgesics have adverse effects, are contraindicated for certain patients, and are effective in pulpitis patients less than half the time [198]. Further investigation into pain pathways and alternative therapeutics are warranted. A key limitation in dental pain research has stemmed from a lack of validated behavioral models to associate the presence of suspected pulpal nociceptors with pain, and few correlative studies between pain sensation and relevant markers in human patients. Thus, the goal of this dissertation was to investigate the effects of purinergic signaling on pain activation and modulation, and to validate common behavioral assays for use in identifying dental inflammatory pain. The overall dissertation hypothesis is as follows:

**Overall hypothesis:** Purinergic signaling, through activation of P2X3R, is directly related to pulp inflammatory pain.
The overall hypothesis will be investigated by three specific aims. The specific aims of the body of work presented in this dissertation are as follows:

**Specific Aim 1:** Determine the presence and distribution of purinergic markers in the human dental pulp in health.

**Specific Aim 2:** Delineate the relationship of the purinergic pathway and dental inflammatory pain in the human *ex vivo* model.

**Specific Aim 3:** Develop quantifiable behavioral assessment models of the orofacial area after dental pulp injury in the *in vivo* mouse model.
CHAPTER 2: MATERIAL AND METHODS

Parts of this chapter are (1) currently under submission for publication as See LP, Sripinun P, Lu W, Li J, Lee S, Karabucak K, Wang S, Theken KN, Mitchell CH. The increased purinergic signaling and P2X3 receptors in human dental pulps with inflammatory pain demonstrates sex-dependent differences (2) or were previously published as Rossi HL, See LP, Foster W, Pitake S, Gibbs J, Schmidt B, Mitchell CH, Abdus-Saboor I. Evoked and spontaneous pain assessment during tooth pulp injury. Sci Rep 2020;10(1):2759. Towards the co-author publication, See LP was involved in the experimental design, carrying out the experiments, and contributed to the writing and editing of the manuscript.

2.1 Experimental design

The experimental design can largely be divided into two components: the ex vivo human study component (Aims 1 and 2) and the in vivo mouse study (Aim 3). The study overview for the ex vivo and in vivo components are depicted in Figures 2-1 and 2-2; respectively. Further detail is described in its respective subsection.

Figure 2-1 Ex vivo human study overview. Flowchart depicted workflow for Aims 1 and 2.
**2.2 Aims 1 and 2: Ex vivo human study**

**2.2.1 Study subjects**

Institutional approval (Institutional review board (IRB) Protocol #833399, University of Pennsylvania) and patient consent was obtained following IRB protocol guidelines. The inclusion criteria consisted of patients with a preexisting indication for tooth extraction. Permanent fully-formed and vital teeth with moderate-to-severe dental caries (extending beyond 2/3 of dentin), presenting with or without pain, were collected. Exclusion criteria included necrotic teeth, irreversible pulpitis due to trauma (ie. fracture, cracks), teeth with resorptive defects, and teeth that became grossly fractured during the extraction process.

The carious teeth were allocated into two groups: symptomatic irreversible pulpitis (SIP) and asymptomatic irreversible pulpitis (AIP) based on standard diagnostic criteria [27]. The diagnosis of SIP was confirmed with a cold test where Endo Ice Refrigerant spray (Coltene; Altstätten, Switzerland) was applied to a cotton pellet, placed on the tooth and produced a positive and lingering pain response [27; 36]. This group consisted primarily of patients presenting to the University of Pennsylvania Oral Surgery Department as emergency visits due to dental pain. The AIP group presented without history of dental pain, and with either normal and/or lingering response to the cold test [27]. However, patients in this group may have been taking pain medications for other chronic pain conditions. Healthy teeth, with absence of pain,
dental caries, or other pathology, were collected for control purposes. This group was comprised of wisdom teeth or teeth extracted for orthodontic purposes. Prior to extraction, all study participants filled out a study survey detailing demographics (ie. age, sex, race), their pain, and pain medication history. An additional pain survey was administered to patients presenting for tooth extraction for dental pain. The pain survey detailed pain severity using a 0-100 visual analog scale (current and when the pain was at its worst), pain quality (sharp, throbbing, aching, mixed), and pain distribution. All study participants received standard-of-care dental treatment for their tooth extraction.

2.2.2 Histological processing and analyses

Immediately after extraction, the teeth were rinsed with saline and split longitudinally. Longitudinal grooves were placed using a water-cooled diamond cutting wheel (IsoMet Blade 15LC, 5 in; Buehler; Lake Bluff, IL), and split using a dental hammer and chisel. Pulpal tissues were obtained and immersed in 10% formalin for ≥ 12 hours. The pulpal tissues were washed in phosphate-buffered saline (PBS), immersed in 30% sucrose overnight, embedded into optimum cutting temperature compound (Fisher HealthCare; Houston, Texas) and cryosectioned into 10-µm serial sections. Hematoxylin and eosin staining was performed on every fifth section to determine levels of pathology and inflammation. The slides were examined under light microscope. Each region of the tooth (coronal, middle, apical) was histologically classified into one of four scores (Figure 3-1), according to the most severe inflammatory changes observed. The sum of the regions provided the cumulative histological score. Slides were examined by 2 masked evaluators. Evaluations were performed separately and scores averaged if different between the two evaluators.
The histological scoring rubric with representative images can be found in Figure 3-1. The scoring rubric is based on slight modifications to previous histological criteria [213; 227]. A score of 1 was histologically diagnosed as healthy pulp. This score was given to dental pulps where cells appeared unaltered. There is absence of inflammatory cells, dilated blood vessels, and calcifications. The healthy pulp is composed of fibroblasts dispersed along a loose connective tissue background. A score of 2 represented initial inflammatory changes and was diagnosed as reversible pulpitis. This was characterized by an increase in collagen fibers and fewer fibroblasts. Calcifications can be seen within the pulpal body. A score of 3 represented more intense inflammatory changes, but maintained the diagnosis of reversible pulpitis. Dilation of blood vessels and edema is evident, where at times extensive heme can be visualized. Typically, three or more blood vessels or neurovascular bundles can be seen in a 20x field-of-view. A score of 4 demonstrated the most severe inflammatory changes and was diagnosed as irreversible pulpitis. This score was given if any area, even if very small, had evidence of liquefactive or coagulative necrosis. The necrotic areas were surrounded by profuse masses of inflammatory cells. Inflammatory cells were identified by their distinct darkly stained nuclei.

2.2.3. Immunofluorescence staining and imaging

Sections were incubated with blocking solution (5% normal goat serum, 1% Triton X-100, 0.5% bovine serum albumin, 0.9% NaCl in 1x PBS) for 2 hours at 25°C and incubated overnight at 4°C with antibodies against P2X3 (1:200; AB5896; Millipore; Burlington, MA), P2X2 (1:200; Ab10266; Abcam; Boston, MA), CD39 (1:200; 188-820; Ancell; Bayport, MN), ADORA-1 (1:200; NB300-549; Novus Biologicals; Englewood, CO),and/or PGP9.5 (1:200; Ab5925, Ab5898; Abcam; Boston, MA) diluted in blocking solution. Sections were washed with
1x PBS and incubated for 1 hour with appropriate secondary antibodies conjugated to Alexa Fluor 488, 546, 555, 568, or 647 (1:500 dilution; Invitrogen; Eugene, OR) at 25°C. DAPI nuclei staining was performed before coverslips were placed with SlowFade™ Gold antifade mountant (Thermofisher Scientific; Eugene, OR). To minimize bias, tissues from one control, SIP and AIP tooth were processed together for each antibody at a given time. Negative controls were performed in parallel in the absence of primary antibodies (Figure 3-S1). 3 to 4 sections per pulp were stained for each antibody to provide a comprehensive and representative overview of the distribution of each protein in health and pulpitis. Entire pulps were imaged using Nikon Eclipse Ti2 Confocal (Nikon USA, Millville NY) and Nikon Elements software using the large-image function at 10x. Additional limited z-stacks were taken to study specific structures at 20x and 40x. Image processing was performed using ImageJ v1.53q [225], with 3-4 sections from each tissue per set of masked control, SIP and AIP tissues analyzed together. The lower threshold was adjusted to remove background, with the same setting used for all tissue sections in a given batch. Positive staining was determined after thresholding and the percentage of area stained was quantified. The average level across the 3-4 sections was taken as the value for the sample.

2.2.4. Western Blot

Pulpal tissue were washed twice with cold Dulbecco's phosphate-buffered saline (Invitrogen; Waltham, MA), and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Cell Signaling; Indianapolis, IN) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, and protease inhibitor cocktail (Complete; Roche Diagnostics, Germany). The samples were sonicated and cleared by
centrifugation (14,000g) for 15 min at 4°C. The protein concentrations were determined using a BCA Protein Assay (Pierce/Thermo Fisher; Rockford, IL). Homogenate containing 15 μg of protein was separated using conventional SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Nonspecific binding was blocked with 5% goat serum in 5% nonfat dried milk for 1 h at 25°C. Blots were then incubated with antibodies against P2X3 (1:1000; ab10269; Abcam; Boston, MA), P2X2 (1:1000; ab10266; Abcam; Boston, MA), or CD39 (1:1000; 188-820; Ancell; Bayport, MN) overnight at 4°C followed by incubation with either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 dilution; Amersham Biosciences Corp., Arlington Heights, IL; ab7090; Abcam; Boston, MA) at 25°C for 1 h. Blots were developed using the chemiluminescence detection (ECL detection system; Amersham Biosciences Corp.; Arlington Heights, IL) and visualized with the ImageQuant LAS 4100 imager and Image Quant software (GE Healthcare Life Sciences; Chicago, IL).

2.2.5. Cell culture

The human retinal pigmented epithelial (ARPE-19) cell line (American Type Culture Collection; Manassas, VA) were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium with 3 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 mg/ml fungizone (all from Invitrogen; Carlsbad, CA), and 10% fetal bovine serum (Corning Mediatech, Inc; Woodland, CA) as previously described [29] until confluent. After cell confluency, fetal bovine serum was reduced to 1%, and cells were subsequently grown for an additional week.
Primary rat neuronal cultures were prepared from embryonic day 18 Sprague–Dawley CD rat embryos (Charles River Laboratories, Wilmington, MA, USA). Brains were isolated, cortices dissected and incubated for 25 min in HBSS containing 2.5% Trypsin (Invitrogen, Waltham, MA, USA) and 80 U/mL DNase (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C. Cortices were then rinsed twice with HBSS, mechanically disaggregated in DMEM + 10% FBS, passed through a 70 μm strainer and resuspended in neurobasal medium supplemented with B27 and GlutaMAX (all from Thermo Fisher Scientific, Waltham, MA, USA). Cells were plated on poly-L-lysine (Peptides International, Inc., Louisville, KY, USA) coated 6-well plates at a concentration of 250,000 cells/mL. Cultures were maintained in neurobasal media with B27 supplement at 37 °C with 5% CO2. Neurons comprised >90% of the total cells under these culture conditions [90] and were used 15 days after plating.

100 μM ATPγS (Cayman Chemicals; Ann Arbor, MI), a nonhydrolyzable ATP analog, in growth medium was applied to cells for a total of 48 h. At 24 h, the medium was replaced with fresh ATPγS solution. For control wells, solutions were replaced with respective growth medium alone at the same time points.

2.2.6. Quantitative PCR (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen; Waltham, MA), purified with the PureLink RNA Mini Kit (Invitrogen; Waltham, MA) and converted to 1 μg (ARPE19 cells) and 500 ng (rat neuroglial cells) of cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Waltham, MA). qPCR was performed with PowerUp SYBR green (Applied Biosystems; Waltham, MA) on the QuantStudio 3 Real-Time PCR system (Applied Biosystems; Waltham, MA) using standard annealing and elongation protocols, with data
analyzed using the delta-delta CT approach as previously described [54]. Primers used are summarized in Table 2-1. Levels of NTPDase1 (CD39) along with other cell-surface located ectonucleotidases (NTPDase2, 3, 8) were examined with GAPDH used as an internal control.

Table 2-1 List of qPCR primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>GGTGTGAACCATGAGAAGTATGA</td>
<td>GAGTCCTTCCACGATACCAAAG</td>
</tr>
<tr>
<td>hNTPDase1 (CD39)</td>
<td>CAGGGACCATGCTTTTCATCC</td>
<td>GCTGGAATGGAAGGTCATCTCA</td>
</tr>
<tr>
<td>hNTPDase2</td>
<td>GCTGGAATGGAAGGTCATCTCA</td>
<td>GAGCTGAAGTCTGTGCCCCTT</td>
</tr>
<tr>
<td>hNTPDase3</td>
<td>GCTCCGCACAGCTAGGAG</td>
<td>TTCTTGAGACCCGACATCC</td>
</tr>
<tr>
<td>hNTPDase8</td>
<td>GCCAGCCCAGGAACCAG</td>
<td>CAGGAGGAATGAGTGCCCG</td>
</tr>
<tr>
<td>rNTPDase1 (CD39)</td>
<td>AGGAGCCTGAAGGCTACCC</td>
<td>GTCTGATTTAGGGCAGGAA</td>
</tr>
</tbody>
</table>

2.2.7. Data analysis and materials

All materials were from Sigma-Aldrich Inc. (St. Louis, MO) unless otherwise noted.

Statistical tests were performed using GraphPad Prism 9. Analyses were performed in a masked fashion whenever possible. The Chi-squared test was used to analyze differences between the groups of normal pulp, SIP, and AIP in terms of sex, race, and medication usage; and differences between male and female ratings of pain characteristics. One-way ANOVA was used for the continuous variables of age and Mann-Whitney test for pain scores. Immunofluorescent and immunoblot analyses were analyzed by two-way and one-way ANOVA, respectively, followed by Tukey’s multiple comparisons test. qPCR analysis was performed with unpaired t-tests. Analyses were performed on ranks when data were not normally distributed. Additionally, bivariate associations between variables were assessed using the Spearman correlation (r). Data was further stratified based on sex and 2-way ANOVA with Sidak’s multiple comparison test performed. Results with p < 0.05 were considered significant.
2.3 Aim 3: In vivo mouse study

2.3.1 Animals

For behavior studies, male and female adult wild type mice consisting of a mixed CD1 and C57BL6/J background were used. Mice were 17–21 weeks old at the time of testing. 27 total mice were used in this study and purchased from the Jackson Laboratories and Charles River. 12 mice total were used for the initial behavior experiments with 6 males and 6 females, which allowed us to make the claims about no sex differences at baseline.

For injury, we randomly separated the groups and 2/6 females and 4/6 males received the DPI surgery, while 4/6 females and 2/6 males received the sham surgery. To evaluate the effect of standard analgesia on the DPI model 10 additional male mice were used. Mice were maintained in a standard 12:12 light dark cycle (lights on at 07:00) tested within a time range of 08:30–13:00. Mice had access to food and water ad libitum when not being tested. All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC Protocol #806515) and follow the guidelines established by the National Institutes of Health.

2.3.2 Dental pulp injury

Mice were anesthetized with ketamine/xylazine (i.p. 100 mg/kg and 12.5 mg/kg respectively) and positioned under a dissecting microscope and warming pad on their back, with their head supported at an angle, and their mouth propped open with forceps. After trimming the oral whiskers, the upper first maxillary molar was drilled on one side using 1/4 round carbide bur until the enamel and dentin layers were breached and the pulp was exposed. This process took about 5 minutes. Enamel appears hard and white, dentin is gray, and when the pulp is visible
vasculature and white to pink tissue can be seen in the cavity under the microscope. Sham animals underwent the same anesthesia, positioning and oral manipulation, but their teeth were not drilled. Moist food was provided for the first 3 days following the procedure and body weight monitored daily during behavioral testing. The percentage of weight loss did not differ between the two groups at the beginning or at the end for the testing period (at 24 hours: DPI - 2.9 ± 0.7% and Sham -3.5 ± 1.2% and at 6 days: DPI -3.3 ± 1.3% and Sham -3.8 ± 1.2%). Mice were either used for behavioral testing on days 1–6 post procedure (n = 6/treatment group), or were immediately euthanized on day 1 (n = 3 injured n = 2 naïve + 1 sham) to collect tissue. The same set of mice was used for Mouse Grimace Scale and Von Frey testing, performed on alternating days.

2.3.3 Anti-inflammatory analgesic treatment

To evaluate alleviation of pain by standard analgesia, ten C57bl/6J males underwent the dental pulp injury procedure as described above. Half of the mice received meloxicam (s.c. 5 mg/kg) the day of the procedure and the next two days twice daily, while the other half received saline on the same schedule. These mice underwent behavioral testing in a treatment-blind manner, including Von Frey at two days post procedure (between drug treatments), and at three days post-procedure (following the end of drug treatments).

2.3.4 Mouse grimace scale

Mice (6/treatment group) were acclimated in the chambers at least twice prior to baseline testing, and were in the chamber for 10 minutes before recording began each day. Before the procedure and on days 1, 3, and 5 after injury, we video recorded mice for 10 minutes in clear
acrylic chambers (4.3 W × 4.3 H × 11 L cm) on a mesh platform from the small end of the chamber with a camcorder (Sony, HVC) with digital zoom. A 3-way mirror was placed at the back of one end to facilitate assessment of unilateral grooming and to prevent the mouse from viewing the next acclimating mouse. From the 10-minute video, one still image for every second of video was extracted using Video to Picture Converter Software (Hootech). From these ~600 images, 10 were selected that contained a clear view of the animal’s face. All of the 480 selected baseline, sham, and post-pulp exposure images were cropped to show only the face and randomized for scoring in a Power Point file. Scoring of the images was performed blind to day and treatment, as indicated in the original method for 5 action units (orbital tightening, nose bulge, cheek bulge, ear position and whisker change), from 0 (not present) to 2 (very visible), and action units were averaged to arrive at the score for each image [161]. In some cases, the whiskers could not be viewed, so this unit was omitted for the score of that image. Performing the statistical analysis with or without the whisker change action unit did not affect the overall statistical results. For sample images of scores for each action unit, see Figure 2-3.
Figure 2-3 Mouse Grimace Scale. Representative Mouse grimace images for each scored action unit.

2.3.5 Mechanical allodynia assessment by von Frey

For these studies animals were placed in confined chambers with adjustable openings (Figure 4-3A). The chamber dimensions were about 7 cm in all directions, with an opening as wide as 2.5 cm. The animals were acclimated to the chambers once for 30 minutes the day prior to baseline testing. Their natural tendency is to put their face out of the opening when it is wide enough, but they are elevated from the floor, which prevents immediate escape. In this way, the animal can be prompted to present its face for stimulation. Mice were stimulated twice on either side of the face, alternating between sides, aiming for the region including the vibrissae to the point in front of the eyes. The animal’s response was scored from 0 to 4 based on early work in rats with neuropathic injury [70; 258] (see Table 2-2 for score descriptions). “Threshold” was defined as the filament that either produced a score of 3 followed by a response of 2 or more, or
the point the animal was no longer willing to pass its face out of the opening after about 5 minutes. The animals were tested on days 2, 4, and 6 post injury with the full filament series (Baseline Tactile Sensory Evaluators, consisting of 11 graded filaments from 0.008 g to 4 g).

### Table 2-2 Score for responses to Facial von Frey

<table>
<thead>
<tr>
<th>Score</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No response</td>
</tr>
<tr>
<td>1</td>
<td>Orientation to the stimulus or a slower head turn away from the stimulus</td>
</tr>
<tr>
<td>2</td>
<td>A rapid withdrawal that may or may not be followed by a single facial wipe</td>
</tr>
<tr>
<td>3</td>
<td>Attacking or biting the filament or rapid withdrawal followed by 2 facial wipes</td>
</tr>
<tr>
<td>4</td>
<td>A rapid withdrawal with multiple facial wipes</td>
</tr>
</tbody>
</table>

#### 2.3.6 Preparation of tissue for histology

At 1-day post-injury, sham and naive mice were deeply anesthetized with ketamine/xylazine and perfused with cold Phosphate Buffered Saline followed by 4% paraformaldehyde through the heart. Their trigeminal ganglia and either the remaining cranium or just the portion of the mouth containing the teeth and nerve roots were removed. Trigeminal ganglia and mandibular regions were post fixed for up to 4 hours and overnight, respectively. Trigeminal ganglia were placed in 30% sucrose until they sunk (overnight), and then frozen in Neg50 media for cryosectioning (20 μm). Teeth were placed in 10% EDTA for approximately two weeks to decalcify, cryoprotected in 30% sucrose, embedded and cryosectioned (20 μm). All tissues were sectioned on a Leica cryostat onto Superfrost plus slides, in a series of 16 (TGs) or 10 (teeth). Adjacent series of TG sections were selected for in situ hybridization using the RNAScope system for 2 probes. Four sections per left and right TGs from 3 animals were mounted on one slide. One series from the teeth underwent standard hematoxylin-and-eosin staining to visualize injury related alterations in the tissue.
2.3.7 In Situ hybridization with RNAScope

Trigeminal ganglia were prepared using a modified version of the manufacturer’s recommendations for fixed frozen tissues used for fluorescence visualization. Briefly, slides were dehydrated in a graded series of alcohol, peroxidase activity was blocked with hydrogen peroxide, and protease IV was applied to the tissue for 30 minutes at room temperature before undergoing the RNAScope Multiplex Fluorescent v2 assay (ACD). The assay was performed according to the manufacturer’s protocol using two probes as we have previously described [207]. TG sections were assessed for overlap between Tlr4 (channel 1) and either Trpa1, Trpv1, or Mrgrpdr (channel 2). Channel 1 was visualized using opal dye 520 and channel 2 was visualized with opal dye 570 (1:1500 for both dyes). Tissues were imaged on a Leica SPE TCM using the same laser power and gain settings for all slides. Because we did not know the time course of pain changes in our DPI model, we opted to leave the pulp exposed, rather than applying a dye after exposure and sealing it and the injury site. Thus, we could not be fully certain that the neurons we visualized in the trigeminal ganglion came from the tooth pulp versus other trigeminal tissues. However, our own preliminary studies and others [146] have shown that maxillary molar labeling with the DiI paste Neurotrace (Invitrogen) results in positive cells in all branches of the trigeminal nerve, therefore cell clusters observed in both the region where V3 and V2 meet, as well as the region where V1 and V2 meet, were imaged resulting in 2 images per section with the 20x objective. As previously described [207] all cells with detectable signal were selected for quantification and the signal intensity of mRNA clusters observed within each cell was analyzed by drawing a region of interest around each cell and mean signal intensity in arbitrary units generated by ImageJ software was noted. The dimensions of the region of interest were kept constant throughout the analysis to avoid bias. This process was repeated for each
channel including overlay images. The entire quantification was performed by an observer in a manner blinded to mRNA probes and channel assignments.

2.3.8 Statistical analyses

Data were assessed for normality using the Shapiro-Wilkes test. Raw Von Frey thresholds were log-transformed to achieve normality so that parametric statistical tests could be used. For Von Frey and Grimace behaviors, two-way ANOVA with repeated measures and between subjects effects were used to determine if there were any significant effects of time, treatment, or a significant interaction, followed by with post-hoc Dunnett’s and Sidak tests where appropriate. For fluorescence intensity, an unpaired student’s t-test was used and **** represents a p-value < 0.0001. Statistical tests were performing using Graph Pad Prism (v8.3).
CHAPTER 3: THE INCREASED PURINERGIC SIGNALING AND P2X3 RECEPTORS IN HUMAN DENTAL PULPS WITH INFLAMMATORY PAIN DEMONSTRATES SEX-DEPENDENT DIFFERENCES

This chapter is currently under submission for publication as See LP, Sripinun P, Lu W, Li J, Lee S, Karabucak K, Wang S, Theken KN, Mitchell CH. The increased purinergic signaling and P2X3 receptors in human dental pulps with inflammatory pain demonstrates sex-dependent differences.

3.1 Results

3.1.1 Patient demographics and pain survey

A total of 95 patients were consented to the study; however, 42 of the patients were deemed ineligible as they did not fit the inclusion criteria, the tooth fractured, or the patient decided against undergoing the extraction. 53 of the enrolled patients were eligible and 54 extracted teeth were collected. The variables of age, sex, race, and pain medication are listed in Table 3-1. Statistical analyses revealed no differences in these variables between the 3 groups, except for pain medication. Pain medication usage was found in all groups but most prominently in the SIP group (p=0.04). Age was trending significance with patients in the AIP group older than those of the control group (p=0.07).

<table>
<thead>
<tr>
<th>Table 3-1 Patient demographics</th>
</tr>
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<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Sex</td>
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</table>

...
The patients presenting to the emergency clinic and diagnosed with SIP characterized their pain as severe with a median current pain score of 72 out of 100 (IQR 59.5), and median worst pain score in that tooth recently as 100 out of 100 (IQR 11.5). Their pain was mostly described as sharp and throbbing in nature with a tendency for referred pain to other areas of the head and neck. The summary of the pain survey can be found in Table 3-2. There were no significant differences in pain intensity, quality, or analgesic usage between men and women.

### Table 3-2 Pain survey of patient with symptomatic irreversible pulpite (SIP)

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients</th>
<th>Men</th>
<th>Women</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current pain (0-100) Mean (IQR)</td>
<td>72 (59.5)</td>
<td>75.5 (8.25)</td>
<td>50 (65)</td>
<td>0.67</td>
</tr>
<tr>
<td>Worst pain (0-100) Mean (IQR)</td>
<td>100 (11.5)</td>
<td>100 (0)</td>
<td>100 (12)</td>
<td>0.14</td>
</tr>
<tr>
<td>Pain medication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>0.29</td>
</tr>
<tr>
<td>No</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Pain quality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharp</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Throbbing</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>0.33</td>
</tr>
<tr>
<td>Aching</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pain distribution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0.13</td>
</tr>
<tr>
<td>Diffuse</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Referred pain</td>
<td>8</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
3.1.2 Histological analyses confirmed that inflammation was limited to SIP and AIP groups

All pulpitis samples demonstrated histological changes consistent with inflammation. Dilated blood vessels and increased neurovasculature were usually present, with the majority of tissues also showing limited areas of necrosis and infiltration of inflammatory cells (Figure 3-1D). Sections were scored from 1-4 based on standardized criteria as shown in (Figure 3-1B,C; see Methods). A significant increase in inflammatory score was noted between tissues diagnosed with either SIP or AIP as compared to healthy control tissues (Figure 3-1E1). Histologically, the greatest inflammatory changes were commonly noted coronally, and reactions would become less severe further away from the area of injury. Typically, the lowest inflammatory scores were seen in the apical portions of teeth (Figure 3-1E2, 3-1E3). The level of inflammation did not differ between the two pulpitis groups, however. Additionally, no significant differences in inflammatory scores were found between men and women in all three groups (Figure 3-1E4). These histological analyses confirm the clinical diagnoses that SIP shows pain with inflammation, AIP inflammation without pain, and controls show neither inflammation nor pain.

3.1.3. Expression of purinergic proteins under control conditions

Previous studies have established the presence of P2X3R immunoreactivity in the human dental pulp [2; 68]; however, co-expression with P2X2R and changes in their relative expression with pain or inflammation have not been examined. Given the role of both the homomeric P2X3R receptor and the heteromeric P2X2/P2X3 receptor in pain transmission [3], we thus examined the expression and distribution of P2X3 and P2X2 receptors in the human dental pulp using immunofluorescence staining.
Initial studies established levels of relevant protein under baseline conditions. In control tissues, P2X3R immunoreactivity was observed throughout the dental pulp on nerve fibers and odontoblasts (Figure 3-2A). P2X2R immunoreactivity was likewise observed throughout the dental pulp on nerve fibers and was frequently colocalized with P2X3R (Figure 3-2B). ADORA-1 immunoreactivity was observed throughout the pulp on PGP9.5 positive sensory nerve fibers (Figure 3-2C).

To screen the use of cell-surface-located ectonucleotidases as an index of ATP release, qPCR was used to evaluate the molecular changes accompanying increases in ATP availability. *NTPDase1* (CD39) and *NTPDase8* were significantly elevated in human retinal epithelium cells after 48 h exposure to ATPγS (*p*<0.001). This elevation was not found with other ectonucleotidases: *NTPDase2* (*p*=0.40), and *NTPDase3* (*p*=0.1) (Figure 3-3A). *CD39* was further examined as an index of ATP availability in primary rat cortical neuroglial cultures, with increased relevance for the dental pulp composition. CD39 was significantly elevated in rat cortical neuroglial cells after 48 h exposure to ATPγS (*p*=0.002) (Figure 3-3B). Using CD39 as an indirect measure of ATP availability, CD39 expression and distribution in the dental pulp were further investigated. CD39 was observed throughout the healthy dental pulp. Expression was greatest on blood vessel-like structures, in proximity with and on nerve fibers, and in the subodontoblast region (Figure 3-3C). It is frequently found in proximity to P2X3R, subjacent to P2X3R-positive odontoblasts and surrounding P2X3R-positive nerve fibers (Figure 3-3D).
3.1.4 **Increased expression of P2X2R and P2X3R in tissues associated with both pain and inflammation**

In the inflamed SIP and AIP tissues, P2X3R expression was found in larger nerve bundles or neurovascular complexes. These bundles were in AIP samples, but widespread in SIP samples, with staining concentrated near the area of injury (Figure 3-4A). P2X3R had significant increased expression in SIP samples compared to both healthy control (p=0.03) and AIP (p=0.04) (Figure 3-4B). The AIP group displayed the lowest expression of P2X3R (no significant difference). The greatest changes in P2X3R expression were observed in the coronal region, the frequent area of injury (Figure 3-4B, p<0.001). This quantitative data was further verified by Western blot. Multiple bands were found with the reduced P2X3R immunoblot (Figure 3-4C), as previously described by other investigators [23; 33; 83]. Most notable were 2 bands located at 50 and 65 kDa, likely corresponding to the major glycosylated forms of the P2X3 receptor [33]. Densitometric measurements revealed significant increases of the 50 kDa band in the SIP group compared to control (p=0.03; n=3) (Figure 3-4D). Under nonreducing conditions, the anti-P2X3R antibody detected a single band, corresponding to the trimeric form of the receptor at ~150 kDa (Figure 3-4C) [61; 79] with increases in the trimeric band for P2X3R in the SIP group.

In SIP and AIP samples, P2X2R expression was more intense and widespread (Figure 3-5A). The expression of P2X2R was significantly increased in SIP as compared to healthy control (p=0.03). There was a non-significant trend towards increased P2X2R staining in the AIP compared to healthy control (Figure 3-5B). Like P2X3R, P2X2R expression had the greatest rise in expression at the area of injury, most commonly the coronal region, with decreasing expression towards the apical region (Figure 3-5B). The P2X2R immunoblot revealed a single
band at 52 kDa (Figure 3-5C). Densitometry of the immunoblot revealed a significant increase in this P2X2R band in the SIP group compared to control (p=0.004; n=3) (Figure 3-5D).

No significant differences in ADORA-1 expression were found between control samples and those with pain or inflammation (p=0.19; Figure 3-S2A). Additionally, ADORA-1 had relatively similar expression levels throughout the different regions of the pulp for all conditions (Figure 3-S2B).

3.1.5 *CD39 is increased in tissues associated with both pain and inflammation*

Samples associated with increased pain and inflammation of SIP exhibited an increase in CD39 expression throughout the dental pulp as compared to both healthy control tissues (p=0.002) and AIP tissues (p=0.007), tissues associated with inflammation but no pain (Figure 3-6A, 6B). CD39 was most highly expressed near the area of injury in the coronal region, and occurred in proximity to P2X3R-positive nerve fibers, with decreased expression moving apically (Figure 3-6B, 6C).

Additional Western blots were performed to confirm the quantitative data. Under nonreducing conditions, two bands were noted for the CD39 immunoblot corresponding with sizes for monomeric and dimeric CD39 (75 kDa and 150 kDa; [38; 45; 84]) (Figure 3-6D). The immunoblot revealed similar levels for the monomeric band, but increases in the dimeric band for CD39 in the SIP group. When proteins were run under reducing conditions, a single band at 54 kDa was detected, corresponding to the C-terminal portion of CD39 [45; 76; 78] (Figure 3-6D). Densitometric measurements revealed significant increases of the 54 kDa band for CD39 in the SIP group compared to control (p=0.02; n=3) (Figure 3-6E).
3.1.6 Sex-dependent and age-associated differences in purinergic markers with pain

Sex differences are well established in clinical pain [69; 84] and dental injury likewise has shown sex dependent changes [37]. To determine whether there were sex-dependent purinergic differences, data was stratified based on sex to evaluate the effect on proteins involved with purinergic signaling. Analyses focuses on the coronal region where there was the greatest levels of inflammation (Figure 3-1E2, 3-1E3) and greatest differences of cumulative receptor expression (Figure 3-4B, 3-5B, 3-6C). A significant difference in CD39 and P2X3R expression was found between men and women in SIP (p=0.002, p=0.01; respectively) (Figure 3-7). Direct comparisons of the total scores were additionally investigated and demonstrated significant differences in CD39 between men and women in SIP (p=0.004, Figure 3-S3I).

Age-related changes have also been shown to affect pain perception [43]. To determine any age-associated effect, Spearman correlation was conducted to examine the association between age with inflammation, pain, and receptor expression. Age had a moderate positive correlation with inflammation in the healthy control group (r=0.59, p=0.02; Table 3). This was not evident in SIP (p=0.22) or AIP (p=0.46), suggesting age to be a factor affecting inflammation in non-diseased pulp. Age was not associated with pain levels, current or worst (p=0.51, p=0.94; respectively; Table 3-S1). There was however a moderate negative correlation of age with P2X3 expression in SIP (r=-0.54, p=0.04; Table 3-3).

Table 3-3 Correlation analyses of age

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th>SIP</th>
<th></th>
<th>AIP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>p-value</td>
<td>r-value</td>
<td>p-value</td>
<td>r-value</td>
<td>p-value</td>
</tr>
<tr>
<td>Age-CD39</td>
<td>-0.06</td>
<td>0.82</td>
<td>-0.19</td>
<td>0.51</td>
<td>0.27</td>
<td>0.32</td>
</tr>
<tr>
<td>Age-P2X3</td>
<td>0.23</td>
<td>0.41</td>
<td>-0.54</td>
<td>0.04</td>
<td>-0.38</td>
<td>0.17</td>
</tr>
<tr>
<td>Age-P2X2</td>
<td>-0.04</td>
<td>0.88</td>
<td>-0.02</td>
<td>0.95</td>
<td>0.10</td>
<td>0.72</td>
</tr>
<tr>
<td>Age-ADORA-1</td>
<td>0.15</td>
<td>0.59</td>
<td>0.31</td>
<td>0.27</td>
<td>-0.07</td>
<td>0.81</td>
</tr>
<tr>
<td>Age-Inflammation</td>
<td>0.59</td>
<td>0.02</td>
<td>-0.34</td>
<td>0.22</td>
<td>-0.21</td>
<td>0.46</td>
</tr>
</tbody>
</table>
3.1.7 Correlation analyses of inflammation, pain, and purinergic markers

Additional Spearman correlation was conducted to study the relationship between inflammatory status and receptor expression, and examine any inter-relation between expression of the four receptor types. There was a moderate positive correlation between CD39 expression and inflammatory score in both SIP and AIP groups (p=0.002, p=0.007 respectively; Table 3-4). P2X2R exhibited a low positive correlation to inflammation in SIP (p=0.019). P2X3R additionally had a trending low positive correlation to inflammation in SIP (p=0.07).

We observed several positive correlations between the purinergic markers (Table 3-5). There was a moderate positive correlation between P2X3R and P2X2R expression in control (r=0.37; p=0.016), SIP (r=0.41; p=0.005) and AIP tissues (r=0.38; p=0.009). Likewise, expression of CD39 and P2X2R were positively correlated in control (r=0.41; p=0.006), SIP (r=0.45; p=0.002) and AIP tissues (r=0.44; p=0.002). A moderate positive correlation was noted between P2X3R and ADORA-1 in both the healthy pulp (r=0.42; p=0.004) and SIP (r=0.39; 0.009). Correlations were only detected in SIP tissues between P2X2R and ADORA-1 (r=0.45; p=0.002) and between CD39 and P2X3R (r=0.46; p=0.002).

Table 3-4 Correlation analyses of inflammation

<table>
<thead>
<tr>
<th></th>
<th>SIP</th>
<th></th>
<th>AIP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>p-value</td>
<td>r-value</td>
<td>p-value</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD39</td>
<td>0.444</td>
<td>0.002</td>
<td>0.397</td>
<td>0.007</td>
</tr>
<tr>
<td>P2X3</td>
<td>0.278</td>
<td>0.065</td>
<td>0.040</td>
<td>0.794</td>
</tr>
<tr>
<td>P2X2</td>
<td>0.347</td>
<td>0.019</td>
<td>0.236</td>
<td>0.268</td>
</tr>
<tr>
<td>ADORA-1</td>
<td>0.268</td>
<td>0.075</td>
<td>0.022</td>
<td>0.884</td>
</tr>
</tbody>
</table>
Table 3-5 Correlation analyses of purinergic markers.

<table>
<thead>
<tr>
<th></th>
<th>Control r-value</th>
<th>Control p-value</th>
<th>SIP r-value</th>
<th>SIP p-value</th>
<th>AIP r-value</th>
<th>AIP p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD39-P2X3</td>
<td>0.286</td>
<td>0.057</td>
<td>0.456</td>
<td>0.002</td>
<td>0.233</td>
<td>0.124</td>
</tr>
<tr>
<td>CD39-P2X2</td>
<td>0.414</td>
<td>0.006</td>
<td>0.445</td>
<td>0.002</td>
<td>0.443</td>
<td>0.002</td>
</tr>
<tr>
<td>CD39-ADORA-1</td>
<td>0.442</td>
<td>0.002</td>
<td>0.361</td>
<td>0.015</td>
<td>0.164</td>
<td>0.280</td>
</tr>
<tr>
<td>P2X3-P2X2</td>
<td>0.370</td>
<td>0.016</td>
<td>0.410</td>
<td>0.005</td>
<td>0.384</td>
<td>0.009</td>
</tr>
<tr>
<td>P2X3-ADORA-1</td>
<td>0.417</td>
<td>0.004</td>
<td>0.388</td>
<td>0.009</td>
<td>0.005</td>
<td>0.972</td>
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<tr>
<td>P2X2-ADORA-1</td>
<td>0.214</td>
<td>0.174</td>
<td>0.446</td>
<td>0.002</td>
<td>0.193</td>
<td>0.333</td>
</tr>
</tbody>
</table>

Correlation analyses were additionally performed for current pain scores, worst pain scores, and receptor expression levels within the SIP group; however no statistically significant correlations were found (Table 3-S1). Adjustments for sex were made and produced similar results (Table 3-S2, 3-S3).

3.2 Discussion

Purinergic signaling is closely associated with both inflammation and pain [24; 59; 73; 83; 132]. However, inflammation and pain are interrelated conditions especially in the context of inflammatory pain. Interestingly, dental pulp inflammation can pursue with or without pain, providing a unique and accessible model to study the two conditions independently. Using *ex vivo* human tissue, our study aimed to determine whether there were purinergic contributions to this presence or absence of inflammatory pain in inflamed pulpal tissue. Additionally, we evaluated the effects of sex and age on purinergic signaling levels. These data provide novel and
clinically relevant insight to purinergic contributions to inflammation and pain in the human
dental pulp model.

We first determined ATP availability by examining CD39 levels in the dental pulp. CD39 is an ATP-diphosphohydrolase, responsible for a cascade converting ATP into ADP and cAMP, ultimately generating extracellular adenosine [214]. Previous studies suggested CD39 a reliable index of ATP availability [178; 179]. These experiments were repeated in this study using human retinal pigmented epithelial cells and rat cortical neuroglial cells. Of the extracellular ectonucleotidases, CD39 (NTPDase1) and NTPDase8 increased in parallel to increased availability of ATP. This complemented previous studies demonstrating CD39 to linearly increase with extracellular levels of ATP [178]. ATP can directly be released from lysed cells, and inflammatory, neuronal, and endothelial cells [83]. Under physiologic conditions, extracellular ATP is present in very low concentrations (400-1000 nM) [219]. However, with inflammation there is marked increase in extracellular ATP levels [24], where ATP acts as a Danger Associated Molecular Pattern (DAMPs) and further induces inflammatory and nociceptive signaling through the activation of purinergic receptors [38; 135].

CD39 has previously been shown to be present and functional in the healthy human pulp. Enzyme histochemistry has shown ecto-ATPase activity in odontoblasts, subodontoblastically, in nerve bundles and large blood vessels within the dental pulp [174; 175]. Due to the available purinergic machinery, it has been proposed that purinergic signaling influences inflammation and pain within the dental pulp. A subsequent study found pulpal upregulation of connexin 43 during pulpitis, further strengthening a role for ATP in pulpitis [171]. Our immunofluorescent and immunoblot data corroborate these findings with increased CD39 expression in the symptomatic inflamed pulp compared with the healthy or asymptomatic inflamed pulp. This increase was
found in the subodontoblastic region, along blood vessels and nerve fibers, generally in proximity to purinergic receptors within the dental pulp. Additionally, CD39 expression levels were positively correlated with inflammation in both SIP and AIP groups. This suggests that purinergic inflammation is present in both symptomatic and asymptomatic disease states, but seems to be a prominent driver of the symptomatic disease.

Purinergic involvement in pain was further investigated by examining levels of P2X3R, P2X2R, and ADORA-1 in the dental pulp. In all pulpal samples, P2X3R was localized to odontoblasts and nerve fibers. The odontoblastic location may suggest its involvement in odontoblastic functions such as dentin sensitivity, as odontoblasts are located on the outermost periphery of the pulp and are the first to detect external stimuli. Previous studies have similarly found P2X3R in the odontoblastic layer and on nerve fibers [8; 212]; however, functional studies in murine odontoblasts described heterogeneous expression of various P2X receptors in odontoblasts with only P2X4R and P2X7R demonstrating functional activity [165; 234]. Though its function in odontoblasts is controversial, a recent study indicated the P2X3 receptor necessary for sensory transduction of dentinal sensitivity through the Piezo1-Pannexin1-P2X3 axis with activation of P2X3 receptors on nerve fibers [200].

Of interest, throughout the pulp body, we consistently found P2X3R with P2X2R on nerve fibers. This phenomenon may suggest that the heteromeric form P2X2/3R is predominant within the human dental pulp. P2X3R currents have fast desensitization onset and slow recovery. However, heteromerization of P2X3R with P2X2R modifies the desensitizing properties and generates sustained non-desensitizing responses [31]. These kinetics may confer the pulp with its unique pain qualities. Both P2X3R and P2X2R were limited to nerve fibers throughout the body of the dental pulp, which suggests a specificity for pain. Furthermore, both receptors were
significantly increased in the symptomatically inflamed group, most drastically at the area of
injury, advocating a role for P2X3R in painful pulpitis. Our finding agrees with previous studies
demonstrating P2X3R expression within the human dental pulp and the potential discrete roles
for P2X3 or P2X2/3 receptors in pain [8; 60; 212; 238]. With the specific increase of P2X3R or
P2X2/3R in symptomatic pulpitis, it is an attractive analgesic target for dental inflammatory
pain.

Adenosine-1 receptor expression was likewise found along nerve fibers throughout the
body of dental pulp. However, its expression did not have a significant difference among the
different groups. This suggests that the adenosine-1 receptor may or may not serve a definitive
role in dental inflammation or pain modulation. A recent study found that Adenosine-1 receptor
activation inhibited P2X3 receptor mediated ATP currents in the rat dorsal root ganglia [111].
This inhibition may also be present in the trigeminal system, independent of changes in
Adenosine-1 expression. We found several positive correlations among the purinergic markers in
both health and disease. In particular, there was a positive correlation between P2X3R and
ADORAl in healthy tissue and symptomatic disease. This may be due to parallel increases in
ATP and adenosine availability. However, this correlation was not apparent in asymptomatic
disease. It is unclear if this imbalance is related to the patient lack of symptoms. A more
definitive role for adenosine signaling in dental pain and modulation awaits further exploration.

As generally trigeminal and orofacial pain have been found to be more prevalent in
women [108; 177], we stratified our present data by sex to determine if there were sex-based
differences in pain quality or purinergic activity in dental disease. We found more female
patients presenting to the emergency clinic with the diagnosis of symptomatic pulpitis, whereas
several male patients presenting with pain were excluded from the study due to the advanced
diagnosis of pulp necrosis (unpublished data). This was similarly described by Nusstein et al [198]. Nonetheless, no significant differences were found regarding pain severity or quality for painful pulpitis between the 2 sexes. Intriguingly, in symptomatic samples we found a significant increase of purinergic markers CD39 and P2X3R in women compared to men. This indicates greater ATP index and purinergic activity in women, suggesting a pathway for different inflammatory and pain responses in men and women.

Different levels of extracellular ATP have been reported to lead to different inflammatory responses [67; 254]. Previously, sex differences in intracellular ATP have been reported regarding mitochondrial function and basal ATP levels in human blood [65; 71; 235]. However, to our knowledge, no studies have examined sex differences in extracellular ATP levels in inflammation. Sex differences in P2X3 expression have been previously reported [63; 216]. Recent murine experimental data have attributed these sex differences to estrogen regulation of P2X3 activity and expression. These studies have shown that estrogen can either increase [54; 205] or decrease [55; 138; 181] P2X3 levels and nociceptive signaling, depending on the concentration of available estrogen [138; 272]. Recently a clinical study reported P2X3 to be positively associated with pain burden in patients with inflammatory bowel disease, but found no sex difference [103]. However, their study was conducted in a much younger age group (8-17 years; mean age 12.8) where sex hormones may not yet play a prominent role. Further investigation is warranted to determine if there is in fact differential purinergic signaling dependent on sex, as most previous studies have opted for all male or female animals or did not report sex-based analyses.

Age was additionally examined as there are reported age changes in pain threshold [163]. Interestingly, our asymptomatic inflamed group consisted of older patients than the symptomatic
inflamed group, though not statistically significant. This is consistent with previous papers demonstrating a prevalence of older patients experiencing “painless pulpitis” than younger ones [143; 189]. This has been attributed to several factors including less innervation in older teeth and reduction in neuropeptide levels [93; 143; 182]. Though there were no evident correlations between age and inflammation or pain in our study, we found a significant moderate negative correlation between age and P2X3R expression within our symptomatic samples. Similar age-related changes with P2X3R have been reported in mice microglia [63].

3.3 Acknowledgements

We thank members of the Mitchell lab for helpful discussion of this work and comments on this manuscript. Additionally, we thank the Kelly Jordon Sciutto Lab, especially Naela Alboloushi and Elena Alvarez-Periel, for their generous provision of primary rat cortical neuroglial cells. Lastly, we thank the Oral Surgery Department, University of Pennsylvania for their contributions in the collection of dental pulp samples.

3.4 Conflict of interest

The authors declare no conflict of interest.

3.5 Author contributions

L.P.S., P.S., W.L, and C.H.M. designed experiments. L.P.S., S.L., B.K., and S.W. collected the dental pulp samples. L.P.S., P.S., W.L., and J.L. carried out the experiments. L.P.S. and P.S. performed all masked scoring. L.P.S., K.N.T., and C.H.M. conducted the data analyses. All authors contributed to the writing and editing of the manuscript.
3.6 Figures

**Figure 3-1 Histological scoring and results.** (A) Dental samples were analyzed and scored by regions: coronal, middle, and apical; respectively. The cumulative score provided the histological score of the sample. (B) Table summarizing scoring rubric used to histologically diagnose the pulp and evaluate levels of inflammation. (C) Representative images of each score taken at 20x magnification. (D) Representative hematoxylin-and-eosin images taken from each region of the three groups at 10x magnification. (E1) Distribution of the histological score for each group. SIP and AIP samples had significantly higher scores than that of control samples (p<0.0001). No statistically significant difference was found between SIP and AIP samples (p=0.13). (E2-3) For both SIP and AIP, the highest inflammatory scores presented in the coronal region, and tapered down towards the apical region. (E4) No significant differences in inflammatory scores were observed between men and women (p=0.17). All data is displayed as mean ± standard deviation.
Figure 3-2 Distribution of purinergic markers within the healthy dental pulp. Purinergic markers (P2X3, P2X2, ADORA-1) were found on nerve fibers (PGP9.5) throughout the dental pulp body, where P2X3R was mostly found colocalized with P2X2R.
Figure 3-3. Distribution of CD39, a marker of purinergic agonist availability, within the healthy human pulp. (A) Elevated expression of NTPDase1 (CD39) and NTPDase8 was detected in retinal pigmented epithelial cells following 48 h exposure to ATPγS (n=3 wells/group; p<0.001). NTPDase2 and NTPDase3 levels were relatively unchanged. (B) Elevated expression of CD39 was additionally detected in rat cortical neuroglial cells following 48 h exposure to ATPγS (n=3 wells/group; p=0.002). qPCR analyses were performed with unpaired t-tests. Mann-Whitney tests were utilized when data were not normally distributed. Data displayed as mean ± standard deviation. (C,D) CD39 staining (red) was found throughout the pulp on neuronal structures (PGP9.5), as indicated by the white arrows, but more intensely staining non-neuronal structures. (D) P2X3R was found on odontoblastic cells (**) and on nerve fibers. CD39 was commonly found in proximity to P2X3R.
Figure 3-4 P2X3R has increased expression in SIP. (A-B) P2X3 had significantly more widespread staining in SIP compared to control and AIP samples (p<0.001; n=15/group). This was most prominent at the area of injury, most commonly the coronal region, and tapered down moving away from injury. Analyses were performed using two-way ANOVA followed by Tukey’s multiple comparisons test. Data is displayed as mean ± standard deviation. (C-D) Representative immunoblot from reducing and nonreducing conditions. The 50 kDa band was found to be significantly increased in SIP compared to healthy samples (p=0.03, n=3/group). Analyses were performed using one-way ANOVA followed by Tukey’s multiple comparisons test. Data is displayed as mean ± standard deviation.
Figure 3-5 P2X2R has increased expression in SIP. (A-B) Immunofluorescence staining revealed P2X2 to have the most widespread and brightest expression in SIP compared to healthy control and AIP (p<0.0001; n=15/group). This was most evident adjacent to injury and returned to more normal levels further away. Analyses were performed using two-way ANOVA followed by Tukey’s multiple comparisons test. Data is displayed as mean ± standard deviation. (C-D). Representative immunoblot of P2X2 protein (52 kDa). Densitometric measurements found significant increases in SIP compared to healthy and AIP samples (p=0.004, n=3/group). Analyses were performed using one-way ANOVA followed by Tukey’s multiple comparisons test. Data is displayed as mean ± standard deviation.
Figure 3-6 CD39 has increased expression in SIP. (A-B) Immunofluorescence staining revealed CD39 to have the most widespread and brightest expression in SIP compared to healthy control and AIP (p=0.002; n=15/group). This was most evident adjacent to injury and returned to more normal levels further away. Analyses were performed using two-way ANOVA followed by Tukey’s multiple comparisons test. Data is displayed as mean ± standard deviation. (C) In SIP, CD39 (magenta) was found to accumulate in proximity to nerve fibers (PGP9.5, red) and P2X3 (green) throughout the pulp. (D-E) Representative immunoblot from reducing and nonreducing conditions. The CD39 C-terminal, 54 kDa, was found to be significantly increased in SIP compared to healthy samples (p=0.02, n=3/group). Analyses were performed using one-way ANOVA followed by Tukey’s multiple comparisons test. Data is displayed as mean ± standard deviation.
Figure 3-7 Women demonstrated greater purinergic signaling than men in SIP. Purinergic signaling demonstrated enhanced expression in women compared to men. Changes of the coronal region were investigated as it was typically the area of greatest inflammation and difference in purinergic marker expression. 2-way ANOVA directly comparing male to female scores of coronal purinergic expression found statistically significant differences in CD39 and P2X3R expression in SIP, where women had heightened purinergic expression compared to men (p=0.002, p=0.01; respectively). Analyses were performed using two-way ANOVA followed by Sidak’s multiple comparisons test. Data is displayed as mean ± standard deviation.
3.8 Supplementary

**Figure 3-S1 Immunofluorescence secondary only controls.** Secondary only controls were performed for all secondary antibodies used in this study. No nonspecific staining were noted with the secondary only controls.
Figure 3-S2 ADORA-1 had similar expression among all groups. (A-B) Immunofluorescence staining revealed no statistically significant differences in ADORA-1 expression between groups (p=0.18; n=15). There was a trend for higher ADORA-1 expression in healthy controls compared to SIP and AIP. Analyses were performed using two-way ANOVA followed by Tukey’s multiple comparisons test. Data is displayed as mean ± standard deviation.
Figure 3-S3 Sex differences in purinergic signaling. Purinergic signaling was enhanced in female samples (A-D) compared to male samples (E-H). Both CD39 and P2X3 showed enhanced expression in women compared to men in the total and coronal region scores. 2-way ANOVA was performed directly comparing female total to male total purinergic scores (I-L). A statistically significant difference was found for total CD39 expression, with greater CD39 expression in women than men in SIP (p=0.004). Analyses were performed using two-way ANOVA followed by Sidak’s multiple comparisons test. Data is displayed as mean ± standard deviation. Further analyses investigated the coronal region (Figure 6).
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4.1 Results

4.1.1 Morphological and gene expression changes twenty-four hours after tooth pulp exposure.

In order to study inflammatory tooth pain, we used the dental pulp injury (DPI) model that we have previously described [97] in which the dental pulp of one maxillary molar tooth is mechanically exposed using a dental drill, producing pulpitis. We began our first analyses 24 hours post-injury, and confirmed controlled removal of enamel and dentin and exposure of the pulp occurred in the molars of DPI-treated mice (Figure 4-1A,B). Foreign material was microscopically present in the tooth cavity of all 3 injured molars, sometimes in contact with the pulp (material next to arrow in Figure 4-1B), demonstrating that an exposed pulp collects materials from the mouse’s outside environment. Importantly, the pulp was still present and clearly exposed, and not yet necrotic, within the injury site at 24 hours (arrow Figure 4-1B).

Next, we utilized RNA-scope technology for a sensitive read-out of RNA levels in the trigeminal ganglion of genes implicated in both nociceptive and inflammatory responses. The trigeminal ganglion was examined as cell bodies of the primary afferent neurons that innervate the dental pulp reside there [146]. We chose to assess the Toll-like Receptor 4 (Tlr4), transient
receptor potential channels vanilloid 1 and ankyrin 1 (Trpv1 and Trpa1), and the mas-related G protein coupled receptor D (Mrgprd), because all are found in neurons that innervate the dental pulp [57; 58; 172; 188; 260] and could be involved in the development of either spontaneous or mechanical pain in the context of infection and injury. In particular, TLR4 is part of a larger class of receptors that recognize pathogen- and damage- associated molecular patterns (PAMPs and DAMPs) [30], and has known interactions with both TRPV1 and TRPA1 in the context of dental injury [188; 260]. A direct role for Mrgprd in dental injury-related pain has not been established, but is possible given its expression in dental afferents [57] and its role in cutaneous mechanical pain perception [45]. We found that the PAMP/DAMP family member Tlr4 was upregulated in DPI-treated versus sham-treated mice (Figure 4-1C,E,I), as were the associated nociceptive channels Trpv1 (Figure 1C,D,H), and TrpA1 (Figure 4-1C,F,J). However, we did not observe an increase in the mechanosensitive nociceptor marker Mrgprd at 24-hours following DPI treatment, suggesting that gene expression changes of this nociceptive membrane protein may not be driving the earliest phases of pain in the DPI model (Figure 4-1C,G,K).

4.1.2 Mouse grimace scale reveals presence of spontaneous pain beginning one day following pulp exposure.

To assess spontaneous pain-like behavior in freely behaving DPI-treated and sham-treated mice, we moved mice into clear custom-made chambers and recorded video of their faces. Still images were selected from these videos for assessment with the MGS (Figure 4-2A). All mice, regardless of treatment exhibited very low MGS scores at baseline, which was not different between the assigned treatment groups and was not significantly affected by the sham treatment (Figure 4-2). We found a significant increase in the MGS at all post-exposure time
points captured (**Figure 4-2C**), there was a significant effect of time, \( F_{3,30} = 5.776 \), \( p = 0.0031 \), treatment \( F_{1,10} = 18 \), \( p = 0.0017 \), and a significant interaction, \( F_{3,30} = 12.75 \), \( p < 0.0001 \). The MGS features that differed in the DPI-treated group versus sham-treated were the pulling back of the ears, nose and cheek bulging, as well as orbital tightening (**Figure 4-2B**). These results are consistent with the previous report showing that MGS scores are highest for pain emanating from internal organs as opposed to the skin [161], demonstrating that this assessment tool can be successfully co-opted for painful pulpitis. This finding also indicates that mice experience ongoing pain within the first day following pulp exposure that persists throughout the observation period.

### 4.1.3 Mechanical allodynia in the face is fully developed by day 4 post pulp exposure and worsens.

To determine how mice respond to evoked stimuli, von Frey hair filaments were applied to the skin between the whisker pad and eye in DPI-treated and sham-treated mice and we scored the behavioral responses associated with the stimulus. Although our stimulus did not touch the tooth directly, hypersensitivity in the orofacial skin surrounding teeth, could indicate a more widespread trigeminal sensitization. Such hypersensitivity has been demonstrated in rats and is reminiscent of findings in the clinic when treatment complications arise [204; 211]. Additionally, mice had to poke their heads through our custom-made chambers to allow the VFHs to make contact with the facial skin. Using this paradigm, we observed that unilateral exposure of tooth pulp on one molar changes response scores and thresholds for Von Frey stimulation on both sides of the face suggestive of mechanical allodynia (**Figures 4-3, 4-4, 4-5**). The earliest significant change was an increase in response scores across all VFHs averaged together, at day
2 on the contralateral side (Figure 4-3B, Contralateral: a significant effect of time: $F_{3,30} = 6.43 \ p = 0.0017$, a significant interaction: $F_{3,30} = 7.69 \ p = 0.0006$, but no significant effect of treatment alone: $F_{1,10} = 2.272 \ p = 0.1626$). By day 4, response scores are significantly increased in pulp-exposed mice on both sides, which persists on day 6 (Figure 4-3B, Ipsilateral: significant effect of time: $F_{3,30} = 3.302 \ p = 0.0336$, no significant effect of treatment alone: $F_{1,10} = 2.34 \ p = 0.1571$, but significant interaction: $F_{3,30} = 4.836 \ p = 0.0073$). Overall this indicates that injured mice, but not sham-treated mice, exhibit a gradual increase in response scores that is most evident on day 4 and maximal on day 6.

We also examined the threshold where animals either scored a 3 or refused stimulation. Raw thresholds (Figure 4-3C) were log-transformed to better conform to normality (Figure 4-3D) and statistical analysis of transformed data indicated a significant decrease from baseline threshold on both ipsilateral and contralateral sides beginning at day 4 post pulp exposure. On day 6, the thresholds of pulp exposed mice were significantly lower than shams (a significant effect of time: ipsilateral - $F_{3,30} = 6.981 \ p = 0.0011$, contralateral - $F_{3,30} = 6.842 \ p = 0.0012$, of treatment ipsilateral - $F_{1,10} = 7.424 \ p = 0.0214$, contralateral - $F_{1,10} = 4.981 \ p = 0.0497$, and interaction ipsilateral - $F_{3,30} = 5.599 \ p = 0.0036$, contralateral - $F_{3,30} = 4.01 \ p = 0.0164$). To confirm that the observed changes to VFH stimulation were driven by inflammatory pain, we tested an additional 10 mice on the DPI paradigm and randomly gave half of the mice saline and the remaining half the commonly used anti-inflammatory drug meloxicam. We notice that at both time-points tested after DPI treatment, as predicted the vehicle only group developed a lowered mechanosensory threshold to orofacial VFH stimulation, while the analgesic group did not (Figure 4-3E). These results are consistent with our DPI model evoking inflammation, and our read-outs being at least partially driven by inflammatory pain.
In regards to potential sex differences, none were found at baseline for any behaviors, based on t-test between females and males with Welch’s correction. For MGS, females were $0.2183 \pm 0.06901$ and males were $0.3533 \pm 0.0726$, ($t = 1.348$, df = 9.974, p = 0.2076). For the Von Frey transformed threshold, which will be discussed in detail in the next section, females were $1.027 \pm 0.537$ and males were $-0.02641 \pm 0.5075$ ($t = 1.426$, df = 9.968, p = 0.1844). For the Von Frey Score, females were $1.522 \pm 0.09181$ and males were $1.311 \pm 0.1824$ ($t = 1.031$, df = 8.857, p = 0.3299). We conclude that at baseline there are no sex differences in our behavioral measurements, but this not preclude distinct behavioral responses or molecular mechanisms that drive pain hypersensitivity after dental pulp injury between the sexes.

Next, we analyzed our Von Frey data in two different ways guided by the response score and the “break point” built into the assay design. The apparatus allows the mouse to learn over time that their natural urge to explore may result in mechanical stimulation that could be painful, at which point they might choose not to expose their face and avoid stimulation, i.e. the stimulus at which this occurs is their “break point”. In the injured mice, we expect that this may occur with lower filament intensities over time, indicating allodynia. We also expect this may occur in the sham group at the higher stimulus intensities with repeated testing, which would indicate hyperalgesia. There is a large degree of disagreement in the field regarding what filament ranges constitute normally “painful” vs “non-painful” stimulation in the absence of injury or damage, which we have attempted to address for hind paw stimulation with VFHs [3]. Often this determination is made arbitrarily by the investigators based on human perception. Here, we use the Von Frey response scores and the break point to determine what range of filament weights correspond to a “non-painful” versus “painful” range. First, we examined the response scores by the weight of each VFH filament, to determine if they were higher across all filament weights,
which would suggest the presence of both allodynia and hyperalgesia. Pulp exposed mice exhibit increasing response scores over time, particularly at lower filament weights (0.008–0.16 g) on both sides (Figure 4-4A,C), which is not exhibited by the sham mice (Figure 4-4B,D). This seems to indicate mechanical allodynia in the injured group. Because the mice reach the cut off score or begin to refuse stimulation, it is unclear if they also have mechanical hyperalgesia, but would be likely.

Second, we determined the weight of filaments that correspond to the break point (when the mouse takes more than 5 minutes to pass its face out of the opening) for both DPI and sham mice for each testing day. As time following pulp exposure increases, the intensity of the filaments where the mice indicate stimulation should stop becomes lower on both sides (Figure 4-5A,C), such that by day 6 the break point occurs at 0.4 g ipsilateral and 0.6 g contralateral. In contrast, although there is some change in the number of sham mice that tolerate stimulation with filaments above 0.4 g after 7 tests, at least two mice tolerated the entire range of filaments during every test (Figure 4-5B,D). Taken together this indicates that exposure of one tooth pulp results in a progressive development of mechanical allodynia, which is fully realized on day 4 post-exposure and increases in severity by day 6. The fact that we have bilateral effects following a unilateral injury likely indicates that central sensitization is manifest within this time frame.

4.2 Discussion

In this study, we found that unilateral pulp exposure injury in mice to the first maxillary molar resulted in a statistically significant increase in MGS from the first 24 hours onward, which is taken as an indicator of spontaneous pain, but does not rule out a general increase in negative motivational affect. The pulp was still present at this time, but clearly exposed when
examined histologically, supporting that the behavior could capture pain originating from the
dental pulp, modeling pulpitis. Surprisingly, mechanical allodynia, as assessed by Von Frey
filament testing, progressed more gradually, with initial changes in scores observed only on the
contralateral side on day 2 post-injury, significant increases seen on both sides at day 4, and
unwillingness to tolerate filaments above 0.6 g by day 6 post-injury. This work demonstrates that
we have clear, easily identifiable behavioral readouts for trigeminal nociception in the mouse
following dental injury. It is possible that the nocifensive behaviors and avoidance of stimulation
captured by the score and break point in the Von Frey assay may not be due solely to pain that
the mouse may be feeling, but could be related to anxiety or fear evoked during the testing
conditions. Even if this is the case, there is clearly still a greater incidence of these behaviors in
the injured mice as compared to sham controls. This is in line with other literature showing
increased anxiety-like behavior and less food seeking in a high-risk environment after pulp
exposure injury [28]. The fact that we see bilateral effects likely indicates that over the course of
4–6 days changes in the peripheral nervous system have led to central changes and sensitization.
Associated with these behavioral changes, we observed significant increases in transcript levels
of Tlr4, Trpv1, and Trpa1, but not Mrgrpdr, in the ipsilateral TG of injured mice as compared to
controls at 24 hours post-injury. Taken together and in support of previous literature [106; 172],
we believe these findings suggest that Tlr4, Trpv1, and Trpa1, may contribute to early changes
resulting in the presentation of spontaneous pain, as indicated by the MGS. However, this does
not rule out a role for Mrgrpdr in the progressive development of mechanical allodynia that seems
to worsen around 4 days following pulp-exposure injury. Based on existing literature from other
body regions [14; 261], TRPA1 could serve as a bridge between the early signaling indicated
here and a hypothesized later process involving MRGPRD. TRPA1 is highly expressed in
mechanosensitive dental pulp afferents [120]. How changes in the TG lead to central sensitization is not entirely clear, but could also be mediated by TRPA1. Several studies have demonstrated that application of mustard oil, the agonist of TRPA1, to the dental pulp produces nocifensive activity in the jaw muscle, due to central sensitization mediated by enhanced glutamatergic signaling in the medullary dorsal horn [53; 248]. We speculate that prior to the onset of mechanical allodynia there may be paracrine signaling processes in the TG that amplify central input and contribute to the development of central sensitization.

Part of our objective in this study was to establish a time course of behavioral changes associated with tooth pulp exposure injury, which is considered by many to be a translationally relevant model for pulpitis [97; 255]. To our knowledge, this is the first assessment of MGS following tooth pulp exposure injury, and somewhat surprisingly the first for facial Von Frey in mouse with this model as well. MGS and the rat equivalent RGS are significantly elevated following other types of dental pain, including tooth movement [276] and mechanical load injury to the temporomandibular joint (TMJ) [241], but in both of these cases, the elevation in score is transient, likely only corresponding with the presence of acute mechanical load. Our elevation in score does not subside, possibly due to the more invasive nature of the injury and the fact that ongoing inflammation is not being treated. In agreement with this idea, in a chronic model of trigeminal neuropathic pain, significant change in MGS is observed 10 days following the constriction injury [7]. It is possible, however, that there may be site or model specific differences. MGS was only transiently elevated following Complete Freund’s Adjuvant inflammation of the TMJ [13], which does not have ongoing infection occurring in the model. In terms of change in the score, our data reflect a similar to slightly greater increase in MGS as compared to tooth movement [276], and potentially within the lower end of ranges reported for
an exogenous-CGRP migraine model [209] and neuropathic injury of the infraorbital trigeminal nerve [7]. This indicates that the mice are likely in a level of discomfort or pain similar to other experimental pain states. Spontaneous pain is diagnostically associated with irreversible pulpitis, supporting the translational relevance of our findings [204].

In addition to spontaneous pain, greater than 50% of patients with irreversible pulpitis also have mechanical allodynia with percussion of the tooth, and these patients have higher ratings of spontaneous pain than those without allodynia [204]. Facial Von Frey, an equivalent means of testing mechanical sensitivity in rodents is challenging, but not impossible in the mouse. Mechanical allodynia in the face has been examined in other experimental paradigms, but has not been published following tooth pulp exposure injury. Most studies have used rats as the model animal, and only one of these used the exact model we use here, where the pulp is left exposed and not treated with exogenous substances [255]. Our findings are in agreement with this previous work in rats. Tsuboi and colleagues also observed a reduction in threshold both ipsilateral and contralateral to the injury, first detectable at day 3, which worsened at day 5 and persisted at least 3 weeks later [255]. This period of time around day 3 or day 4 seems to mark a transitional state between the acute inflammatory response and development of pathological pain states often associated with chronic or ongoing pain. We speculate that the early change in MGS may be established by either the same or different mechanisms than those that produce mechanical allodynia later.

To begin to address this question, we examined the mRNA expression of Tlr4, trpv1, Trpa1, and Mrgprd using in situ hybridization at 24 hours following pulp exposure injury. A great deal of attention has been paid to TLR4 as a possible drug target for the treatment of inflammatory pain in various parts of the body [30], but particularly in pulpitis given its role in
recognizing molecular signals of bacterial presence and mechanical injury and upregulation in human pulpitis samples [260]. Furthermore, antagonism of TLR4 is associated with reversal of pain-associated behaviors in two different rat models of pulpitis [172; 199], including mechanical hypersensitivity in lightly anesthetized rats [199]. Our findings of increased Tlr4 in the trigeminal ganglia 24 hours following pulp injury suggest an association between the function of this receptor and at least increased malaise or spontaneous pain suggested by increased MGS. We need to directly antagonize TLR4 in the context of pulp-exposure injury to verify causality for increased MGS and determine if early intervention might prevent the delayed presentation of mechanical allodynia. It is possible that TLR4 upregulation begins a cascade of molecular events, as of yet not clearly identified, that establish a change in mechanical sensitivity.

Coinciding with the increase in TLR4 we also observed an increase in Trpv1 mRNA expression at 24 hours post injury, similar to increased protein expression found in rats with pulp exposure or complete Freund’s adjuvant (CFA)-induced pulpitis models [172; 264]. Upregulation of the nociceptive channel TRPV1 has been demonstrated within 24 hours of LPS application to the tooth pulp, but returned to control levels 3 and 5 days later [58]. Furthermore, LPS can directly act on TRPV1+ trigeminal nociceptors via TLR4 signaling [74]. Antagonism of TRPV1 in the CFA model blocks mechanical hypersensitivity in lightly anesthetized rats [264], suggesting that TRPV1 could be involved in the development of mechanical allodynia in our pulpitis model. However, given the delayed progression of mechanical allodynia reported here, it is likely that other events downstream of the increased TRPV1 expression in the ganglia are also involved in the pulp exposure model.
We also observed an increase in the expression of Trpa1 in the ipsilateral TG at 24 hours post-injury. As mentioned, targeting TRPA1 tooth pulp afferents, many of which are mechanosensitive [120], can produce central sensitization [53; 248]. Only one other study has examined protein expression following pulp exposure injury in rat molar [106]. They also observed increased expression of TRPA1, but it was not significant until Day 4 [106]. Our differing results may be due to species differences or may reflect a disconnect between the time to peak mRNA levels versus protein levels. Like TRPV1, there is also evidence for an interaction between TLR4 or LPS and TRPA1-related activity. In the DRG, there is evidence that TRPA1 is required for direct nociceptor responses to LPS, even in the absence of TLR4 [188]. LPS increases the percentage of trigeminal neurons responding to the TRPA1 agonist acyl-isothiocyanate (AITC) as demonstrated by calcium imaging [190]. TRPA1 has been implicated in the development of mechanical allodynia in the lower body [168], and is very clearly involved in central sensitization when TRPA1 is targeted in the dental pulp [53; 248], thus could be involved in the mechanical allodynia reported here.

While we observed increased expression of Tlr4, Trpv1, and Trpa1 24 hours post pulp exposure, we did not observe an increase in Mrgprd, also found in the pulp [57] and directly implicate in cutaneous mechanical nociception [45]. However, this does not completely rule involvement of Mrgprd + trigeminal neurons in the development of delayed mechanical allodynia. Future studies will evaluate the expression of Mrgprd closer in time to the manifestation of mechanical allodynia around day 3 or 4. TRPA1 and Mrgprd have been linked in the development of mechanical allodynia in the lower body [14], so some interaction could be involved in the dental pulp, particularly since TRPA1 is co-expressed in mechanosensitive dental pulp afferents [120]. Alternatively, paracrine signaling in the trigeminal ganglia via gap junction
connections with satellite glia [155; 199; 264] could allow for recruitment of the non-peptidergic TLR4 negative MRGPRD population by the peptidergic TLR4+/TRPV1 and/or TRPA1+ cell populations to contribute to central mechanisms underlying the mechanical allodynia we observed.

Although the model presented here is an inflammatory pain model, the DPI model has clear similarities to the orofacial mental nerve neuropathic (MNI) pain model, as we have previously described [166]. For example, we previously showed that both DPI and MNI induce trigeminal neuron expression of the injury gene activating transcription factor 3 (ATF3) and one week after both of these injuries we observed hypersensitivity to mechanical hind paw stimulation, indicating a central sensitization phenotype [23]. Although the studies here demonstrate that our methods are useful for assessing inflammatory pain in the tooth pulp, future studies are needed to determine if these methods will be useful in measuring pain in neuropathic pain models in the trigeminal region.

4.3 Acknowledgements

We thank members of the Abdus-Saboor lab for helpful discussion of this work and comments on this manuscript. This work was supported by startup funds from the University of Pennsylvania to I.A.S. and the National Institutes of Health (NIDCR) R00 grant (DE026807) to I.A.S.

4.4 Conflict of interest

The authors declare no conflict of interest.
4.5 Author contributions

H.L.R., L.P.S., J.G., B.S., C.H.M. and I.A.S. designed experiments. H.L.R. and L.P.S. carried out experiments, and W.F. and S.P. scored fluorescent intensity of RNA scope images. W.F. administered drug treatments in the analgesia experiment. All authors contributed to the writing and editing of the manuscript.
4.6 Figures

Figure 4-1 Changes in tooth morphology and trigeminal ganglia transcript levels 1 day following pulp injury. (A) Intact first maxillary molar from a sham animal and (B) injured maxillary molar with pulp exposure evident. Dotted outline marks the molar and the arrow indicates the exposure site. Healthy pulp is still evident on either side of the opening and compacted foreign material (next to arrow) was present in the cavity. Arrowhead marks an area showing a potential periapical lesion typically seen in tooth infections. (C) Violin plot of quantified fluorescence intensity measured in arbitrary units (a.u.) defined in ImageJ for TrpV1, Tlr4, TrpA1, and Mrgprd in the trigeminal ganglia of injured (blue, n = 3 mice) or sham/naive (red, n = 3 mice) mice. Data displayed as mean ± standard deviation. (D–G) Representative images of RNAscope in situ hybridization following sham and (H–K) 1 day after dental pulp injury. **** is p < 0.0001 for an unpaired T-test.
Figure 4-2 Mouse Grimace Scale following pulp exposure. (A) Before injury mice have low or no score for each of the action units, while after injury (B) prominent presence of the action units, as labeled on the example images from the same mouse. (C) We found a significant increase in the Mouse Grimace Score at all post-exposure time points captured, n = 6/treatment, Dental Pulp Injury (blue) and sham (red). Square shapes with black borders and lighter data points indicate females. *Indicates p < 0.0001 within the pulp exposed group before vs. after (Dunnett’s post-hoc). Data displayed as mean ± standard deviation.
Figure 4-3 Facial Von Frey apparatus, mechanosensory response scores, and threshold changes following pulp exposure. (A) The mice were placed in the chamber for testing, which has adjustable openings that can be closed between tests and allows mice to put their head past the bars if they choose. When mice choose to expose their faces they are stimulated as depicted with a von Frey hair. Image created at Biorender.com. (B) After dental pulp injury (blue) there is a significant increase in mechanosensory response scores across all of the filaments tested, which is not observed in sham-treated mice (red). This is evident from day 4 onward on the ipsilateral side, and on day 2 onward on the contralateral side. (C) Raw thresholds are significantly different from normality according to the Shapiro-Wilks Test, but a decrease with time is apparent in the injured group not observed in sham mice. (D) After log transformation of threshold data to conform to normality, we find a significant decrease in threshold following pulp exposure on both sides, evident from day 4 and significantly lower than shams at day 6. *Indicates p < 0.05 for the indicated time point versus baseline (Dunnett’s post-hoc). Square shapes with black borders and lighter data points indicate females. (E) Saline treated mice had a significant decrease in their threshold at both time points following the injury, which did not occur in meloxicam treated mice. *Indicate p < 0.05 by Dunnett’s post-hoc test. All males used in panel E. Data displayed as mean ± standard deviation.
Figure 4-4 Mechanosensory response score by filament intensity and day post-injury or sham. (A) Ipsilateral and (C) contralateral response scores for low intensity filaments are increased at day 4 (purple) following injury, and by day 6 (red) mice met threshold criteria or refused filaments higher than 0.16 or 0.4 g. In contrast, (B) ipsilateral and (D) contralateral scores were similar across days post-sham procedure and low filament scores did not increase over time.
Figure 4-5 Loss of mouse participation by stimulus intensity (the break point) before and after injury or sham. On the (A) Ipsilateral and (C) contralateral sides, there is a progressive decrease in the number of injured mice willing to tolerate filaments higher than 0.4 g, such that by day 6 none of them progressed further than 0.16 g (ipsilateral) or 0.4 g (contralateral). In contrast, on the (B) ipsilateral and (D) contralateral sides the sham cohort consistently tolerated testing with lower intensity filaments and at least two mice tolerated all the filaments across all testing days.
CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

5.1 General conclusions

In this dissertation, we expanded on the role of purinergic pathways in the clinical model and validated nociceptive behavioral models for use in preclinical rodent models. In all, the combination of a preclinical model and clinical samples allows advancement in the field of dental and pain research in identifying clinically meaningful targets and routes for more mechanistic investigation preclinically. Results from Chapter 3 implicate purinergic signaling in dental inflammatory pain. Purinergic receptors, P2X3 and P2X2, and our index for agonist availability, CD39, were significantly increased in inflammation with pain, but not in inflammation without pain. Expression of these purinergic markers suggested sex differences, where women had enhanced purinergic signaling and expression compared to men. Results from Chapter 4 demonstrated that common behavioral assessment assays could be successfully applied to the orofacial area to measure dental pain. Namely, the Mouse Grimace Scale (MGS) was a reliable indicator of spontaneous pain, resembling the Faces pain rating scale commonly used in pediatric clinics. Also, the facial von Frey assay was a reliable indicator of mechanical pain, translating to the percussion test commonly used in the Endodontic office. In this section, we will discuss the implication of this work, point to the limitations in this research, and outline future experiments to further elucidate the role of purinergic signaling in dental health and disease.

5.2 Purinergic signaling is increased in symptomatic inflammatory disease

Data presented in Chapter 3 support the hypothesis that ATP release and signaling play a role in dental inflammation and pain in human pulpal tissue. In summary, histology was used to
verify the clinical diagnoses and determine the level and area of greatest inflammation. Immunofluorescence and immunoblot studies revealed P2X3R and P2X2R were specific to dental nerve fibers and selectively increased in symptomatic inflammation, suggesting a specificity for pain. Targeting P2X3 and P2X2/3 receptors, in conjunction with our current analgesic regime, may potentially reduce dental inflammatory pain and promote pain-free dentistry. CD39, an index of ATP release, was upregulated in symptomatic inflammation and positively correlated with disease severity in both symptomatic and asymptomatic inflammation. This supports ATP’s role as a DAMP in dental inflammation with augmented changes in symptomatic disease. Sex differences were implicated with CD39 and P2X3R levels significantly enhanced in women compared to men in symptomatic disease. Additionally, age negatively correlated with P2X3R levels. Overall, these data provide novel information in human dental samples associating purinergic signaling and painful inflammation, and suggesting sex and age be taken into consideration when designing clinical trials with purinergic agents.

The main constraint on this study was the time constraint, affecting the sample size and collection. Additionally, there are effects of natural variations that come with human samples. Though we were able to collect an \( n \) of 15 per group for immunofluorescence studies and an \( n \) of 3 per group for immunoblots and demonstrate statistical significance, this study would have benefited from matched sample donors (ie. Sex, age, medication), exclusion of medication usage as the effects of medication on protein expression are unclear, or a larger sample size to increase the power of the study. Additionally, patients presenting for urgent extractions demonstrated sex differences in pulpal diagnoses, where women more frequently presented with SIP and men more frequently presented with pulp necrosis. This phenomenon was also reported in other studies [198]. This has been attributed to women’s greater concern for oral care or possibly lower pain
threshold. Thus, the timing and possibly more chronic nature of the disease in men may also affect the sex differences found in purinergic expression.

Nonetheless, our study serves a critical step in identifying purinergic involvement and distribution in clinical samples with pain and inflammation, and demonstrating effects of sex and age on purinergic signaling—thus setting precedence for further study and consideration of these biological factors. Future experiments would aid confirmation of our findings and further investigation of the effects of biological factors or pharmacological agents. Future directions include the use of an in vivo model in combination with the dental pulp injury and the behavioral models described in Chapter 4. The in vivo model would allow a more controlled setting for standardization and investigation of biological variables and timing of disease. With genetic manipulation or pharmacologic intervention, the role of purinergic signaling in inflammation and pain can be further investigated along with any interaction with other pathways and/or drugs. Favorable findings may prompt further study by clinical trial, using agents summarized in Table 1-2 compared to or in combination with the current analgesic regime and/or placebo.

5.3 Evoked and spontaneous pain assessment models can identify dental pulp inflammatory pain in mice

The primary goal of Chapter 4 was to optimize the assessment of pain-like behavior in conscious, unrestrained mice following induction of pulpitis. We also sought to determine the onset and duration of these pain-like behaviors during the first six days of pulp exposure. Here we take advantage of existing assays, with some custom modifications, and adapt them for assessment of pain-like behaviors during tooth pulp inflammation. After morphologically confirming our tooth pulp injury model, we used RNAscope technology to determine the time...
course of changes in molecular mediators of nociception in the peripheral nervous system relative to behavioral changes. To the best of our knowledge, this is the first study using the MGS to evaluate nociception following dental injury, and our results revealed the occurrence of spontaneous pain-like behavior within the first day following dental injury. We also adapted previous facial Von Frey methods to evaluate mechanical sensitivity, relying on the published scoring scheme, as well as the animals’ willingness to put its head through a custom designed chamber with an adjustable opening for stimulation. Because the mice can decide if they want to expose their faces to the stimuli, we were also able to record the threshold in which mice are no longer inquisitive enough to tolerate facial stimuli, and this tolerance threshold was able to segregate injured versus sham mice. Interestingly, these two assays present a different time course following injury, indicating putative spontaneous pain early and throughout the 6-day observation period, while mechanical allodynia and stimulation intolerance is delayed. The fact that we note bilateral changes in the response to mechanical stimuli suggests that central sensitization is also occurring, but the relationship between changes we observed in the ganglia and central mechanisms have yet to be uncovered. Taken together, the behavioral assays we have defined here to assess pain-like behavior in the mouse should make it easier for researchers to adopt these approaches to aid in uncovering mechanisms for tooth pain.

A key limitation of previous animal pain research was lack of established, easily accessible, and relatively inexpensive pain assessment models. There was reliance on assumptions such as small neuronal bodies represented nociceptive bodies, and that a positive neuronal response was equivalent to pain output. These assumptions overlook other processes involved in peripheral and central nociceptive modulation that impact the final nociceptive output. Thus, behavioral assessment models offer a key advantage if they can accurately detect
and segregate pain versus non-pain behaviors. Our study shows reliability of MGS and facial von Frey in identifying dental inflammatory pain. The limitation of our study is the limited time points. Our study demonstrates a timeline of up to 6 days where MGS were studied on days 1, 3, 5 and facial von Frey were studied on days 2, 4, 6. Future studies may expand on this timeline to study effects of more chronic disease, optimize the von Frey filaments in a manner where the force used can be more controlled and standardized, or expand uses of these behavioral assays to other nociceptive states ie. Neuropathic pain. Additionally, though we validated assays for spontaneous and mechanical pain, a missing link is an assay for thermal pain. A future direction would be validation of a thermal assay, which would be clinically significant as it mirrors the diagnostic test commonly used in the Endodontic clinic to detect disease.

5.4 Summary

This body of work implicates the presence of purinergic signaling, through P2X3R and P2X2/3R, in clinical dental inflammatory pain, and demonstrates effects of sex and age on purinergic signaling. Modulation of P2X3R and P2X2/3R may offer a viable analgesic alternative for dental pain management. Further investigation was hindered by a lack of behavioral models to detect pain in preclinical studies. Thus, common behavioral assays were investigated and validated for use in detecting dental inflammatory pain in mice. Further study of the purinergic pathway utilizing the behavioral assays described would be beneficial in further elucidation of purinergic signaling in dental pain.
APPENDIX

The following documents are attached in sequential order.

(1) IRB approval letter

(2) IRB application

(3) IRB-approved informed consent

(4) IRB-approved assent form

(5) REDCAP-based pain survey
The above referenced protocol was reviewed and approved by the Institutional Review Board using the expedited procedure set forth in 45 CFR 46.110 on 09-Sep-2020. This study has been determined to pose minimal risk to subjects and be eligible for expedited review category(ies) 3,4,7.

Consistent with the regulations set forth in 45 CFR 46.109(f), continuing review of this research is not required. IRB approval of this protocol will not expire and continuing review applications should not be submitted. However, you are still required to submit modifications and reportable events to the IRB for review.

The documents included with the application noted below are approved:

-HSERA Application, confirmation code: dcccccha, submitted on 9/2/20

Notes/Reminders

The IRB reviewed and approved the Subpart D review as per Federal Regulations 45 CFR 46.404 (FDA 50.51), as the research was determined to be no greater than minimal risk. The IRB determined that permission of one parent is sufficient and that adequate provisions are made for soliciting permission. The IRB has determined that assent must be obtained from subjects and appropriately documented.
NOTE: Approval by the IRB at this time DOES NOT constitute authorization to initiate or continue in-person research during the COVID-19 pandemic. Please review Guidance on Notification to the IRB of In-Person Research Resumption During Phase II (Effective 7/13/2020) on the IRB website here for further details: https://irb.upenn.edu.

ONGOING REQUIREMENTS:

- You must obtain IRB review and approval under 45 CFR 46 if you make any changes to the protocol, consent form, or any other study documents subject to IRB review requirements. Implementation of any changes cannot occur until IRB approval has been given.
- Reportable event, such as serious adverse events, deviations, potential unanticipated problems, and reports of non-compliance must be reported to the IRB in accordance with Penn IRB SOP RR 404.
- When enrolling subjects at a site covered by the University of Pennsylvania’s IRB, a copy of the IRB approved informed consent form with the IRB approved from/to stamp must be used unless a waiver of written documentation of consent has been granted.

COMMITTEE APPROVALS: You are responsible for assuring and maintaining other relevant committee approvals. This human subjects research protocol should not commence until all relevant committee approvals have been obtained.

If your study is funded by an external agency, please retain this letter as documentation of the IRB’s determination regarding your proposal.

If you have any questions about the information in this letter, please contact the IRB administrative staff. A full listing of staff members and contact information can be found on our website: http://www.irb.upenn.edu

***This letter constitutes official University of Pennsylvania IRB correspondence.***
### Protocol Details

#### Basic Info

- **Confirmation Number:** dcccccha
- **Protocol Number:** 833399
- **Created By:** SEE, LILY P
- **Principal Investigator:** MITCHELL, CLAIRE H
- **Protocol Title:** Characterization of Pain Receptor Expression in the Human Dental Pulp
- **Short Title:** Pain Receptor in the Human Dental Pulp
- **Protocol Description:**
  
  We propose to use adult extracted teeth to determine if pain receptors, hMRGPRx1 and P2X, are upregulated in the symptomatic inflamed pulp. Pulpal diagnosis will be confirmed by routine diagnostic tests. Receptor expression in healthy and inflamed pulpal tissue will be investigated using antisera against the receptors and by performing serial section immunostaining on tooth pulp. Additionally, a questionnaire will look at the relationship between patient symptoms and receptor expression.

- **Submission Type:** Biomedical Research
- **Application Type:** EXPEDITED Category 3

#### Resubmission*

Yes

#### Hospital Sites

Will any research activities and/or services be conducted at a Penn Medicine affiliated hospital site?

No

### Study Personnel

#### Principal Investigator

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- **Training Expiration Date:** 03/31/2020
- **Name of course completed:** CITI Protection of Human Subjects Research Training - ORA
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HS Training Completed: No
Training Expiration Date: Name of course completed:

Responsible Org (Department/School/Division):
5110 - Anatomy and Cell Biology

Key Study Personnel
None

Disclosure of Significant Financial Interests*
Does any person who is responsible for the design, conduct, or reporting of this research protocol have a FINANCIAL INTEREST?
No

Penn Intellectual Property*
To the best of the Principal Investigator's knowledge, does this protocol involve the testing, development or evaluation of a drug, device, product, or other type of intellectual property (IP) that is owned by or assigned to the University of Pennsylvania?
No

Certification
I have reviewed the Financial Disclosure and Presumptively Prohibited Conflicts for Faculty Participating in Clinical Trials and the Financial Disclosure Policy for Research and Sponsored Projects with all persons who are responsible for the design, conduct, or reporting of this research; and all required Disclosures have been attached to this application.
Yes

Biomedical Research

Clinical Trial*
Is this a clinical trial?
No

Investigator Initiated Trial*
Is this an investigator initiated trial?
No
Drugs or Devices*
Does this research study involve Drugs or Devices?
No

IND Exemption

For studies that fall under an IND exemption, please provide the number below

For studies including IND or IDE’s, please provide the number(s) below

IDE Review*
NOTE: For research involving investigational devices, you are required to review the guidance on Managing Research Device Inventory. Consult the Penn Manual for Clinical Research: https://www.med.upenn.edu/pennmanual/secure/investigational-product-management-at-sites-not-using-investigational-drug-services-(ids).html Please check the box Yes if you have reviewed the guidance.
Yes

Research Device Management*
Please indicate how research device(s) will be managed.
Not Applicable (no investigational devices)

Drug, Herbal Product or Other Chemical Element Management *
Please indicate how drugs, herbal products or other chemical entities will be managed.
Not Applicable (no drugs, herbal products or other chemical entities)

Radiation Exposure*
Are research subjects receiving any radiation exposure (e.g. X-rays, CT, Fluoroscopy, DEXA, pQCT, FDG, Tc-99m, etc.) that they would not receive if they were not enrolled in this protocol?
No

Gene Transfer*
Does this research involve gene transfer (including all vectors) to human subjects?
No

Human Source Material*
Does this research include collection or use of human source material (i.e., human blood, blood products, tissues or body fluids)?
Yes

CACTIS and CT Studies*
Does the research involve Center for Advanced Computed Tomography Imaging Services (CACTIS) and CT studies that research subjects would not receive if they were not part of this protocol?
No

CAMRIS and MRI Studies*
Does the research involve Center for Advanced Magnetic Resonance Imaging and Spectroscopy (CAMRIS) and MRI studies that research subjects would not receive if they were not part of this protocol?
No

Investigational Agent or Device within the Operating Room*
Does the research project involve the use of an investigational agent or device within the Operating Room?
No

Cancer Related research not being conducted by an NCI cooperative group*
Does this protocol involve cancer-related studies in any of the following categories?
No
Processing of Materials*
Will the research involve processing (such as over encapsulating, or compounding)?
No

In-House Manufacturing of Materials*
Will the research involve processing (such as over encapsulating, or compounding)?
No

Medical Information Disclosure*
Does the research proposal involve the use and disclosure of research subject's medical information for research purposes?
Yes

If the answer is YES, indicate which items is is provided with this submission:
Modified research informed consent document that incorporates HIPAA requirements

CTRC Resources*
Does the research involve CTRC resources?
No

Pathology and Laboratory Medicine Resources*
Will samples be collected by hospital phlebotomy and/or processed or analyzed by any of the clinical laboratories of the University of Pennsylvania Health System?
No

Research Involves Apheresis, Cell Collection, and/or Blood Product Collection*
Does this research involve collection of blood products in the Penn Donor Center and/or the use of apheresis for treatment or collection of cells or other blood components?
No

Research involving blood transfusion or drug infusions*
Will your research involve blood transfusion or infusion of study drug in 3 Ravdin Apheresis Unit for research purposes?
No

Trial in Radiation Oncology
Is this research a prospective trial being done in Radiation Oncology, and if so, has this protocol been approved by the Radiation Oncology Protocol committee?
N/A

Study in Radiation Oncology
Is this research a retrospective study being done in Radiation Oncology, and if so, has this project been reviewed by the Radiation Oncology Clinical Research Group?
N/A

Use of UPHS services*
Does your study require the use of University of Pennsylvania Health System (UPHS) services, tests or procedures*, whether considered routine care or strictly for research purposes?
No

Primary Focus*
Tissue/biospecimen
Protocol Interventions

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The following documents are currently attached to this item:

There are no documents attached for this item.

Sponsors

Business Administrator

none

Department budget code

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Funding Sponsors

Funding sponsors billing address
If you have selected a commercial or industry sponsor, please provide the appropriate address and contact information for the Sponsor for the purposes of billing for IRB review fees (initial review, continuing review and convened modification fees apply here). If the Sponsor is not industry/commercial, this information is not necessary to provide with your application.

Funding sponsors gift
Is this research being funded by a philanthropic gift?

Regulatory Sponsor

IND Sponsor

none

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Industry Sponsor

None

Project Funding®
Is this project funded by or associated with a grant or contract?
No

Sponsor Funding
Is this study funded by an industry sponsor?
Status of contract

The following documents are currently attached to this item:

There are no documents attached for this item.

Multi-Center Research

Penn as lead
1. Is this a multi-center study where Penn is serving as the Lead Site or the Penn PI is serving as the Lead Investigator?
No

Management of Information for Multi-Center Research

Penn irb of record
2. Is this a multi-center study where the Penn IRB will be asked to serve as the IRB of Record for other external study sites?
No

Other Sites

No other sites

Protocol

Abstract
Pain receptors, hMRGPRX1 and P2X, have proven to be involved in persistent inflammatory and neuropathic pain in mice. Currently, chronic pain is an enormous health problem affecting millions in America, especially dental pain. This present study will look at hMRGPRX1 and P2X expression in dental pulp and its relation to dental pain. We will investigate hMRGPRX1 and P2X expression in the normal and inflamed dental pulp. This study may have implications for potential preventative therapies aimed at blocking pulpitis pain, which may circumvent the need for opioids and potentially save teeth in patients who would otherwise elect for tooth extraction.

Objectives

Overall objectives
The overall objective of our studies is to advance our understanding of pain receptor expression in the human dental pulp. Specific Aim 1. To identify if hMRGPRX1 and P2X are expressed in the human dental pulp. Specific Aim 2. To examine hMRGPRX1 and P2X expression in healthy and symptomatically inflamed human dental pulp. Specific Aim 3. To examine the relationship of hMRGPRX1 and P2X expression in the dental pulp to other pain factors (ie. pain stimulus, pain quality, migraines)

Primary outcome variable(s)
None

Secondary outcome variable(s)
None

Background
The tooth contains two tissue types responsible for monitoring the integrity of the enamel barrier and
signaling the presence of infectious agents: the dentin and the primary trigeminal afferents that innervate the tooth pulp. One detrimental consequence of infection and inflammation is intense, debilitating pain, which is ultimately transmitted by the trigeminal afferents. If the inflammation is not controlled, these tissues within the tooth pulp may die and the infection could spread causing resorption of the adjacent bone. Currently, the best therapeutic action is to extract the pulp or the tooth itself. In order to preserve the teeth and eliminate infection, we need to have a better understanding of the molecular mediators responsible for signaling pain from the tooth and how they change during the course of inflammation. As stated previously, the transmission of noxious stimuli in the orofacial region is mediated by primary nociceptive neurons in the trigeminal ganglia (TG). These neurons are broadly classified into several populations based on expression of molecular markers (peptidergic versus non-peptidergic) and other criteria (Zylka et al., 2005). Most non-peptidergic C-nociceptors in TG highly express Mas-related G-protein coupled receptor member D (MRGPRD) and are responsive to noxious stimuli and itch-inducing compounds (Dussor et al., 2008; Liu et al., 2012; Rau et al., 2009). Although ablation of MRGPRD+ neurons reveals a deficit in mechanical pain sensation in the spinal system (Cavanaugh et al., 2009), the role of MRGPRD+ neurons in orofacial pain is completely unknown (Chung et al., 2012). We believe MRGPRD+ neurons may be critical for chronic pain in the orofacial region. Since the discovery of the family of Mas-related G-protein coupled receptors (Mrgpr) nearly twenty years ago, pain researchers have been intrigued with these genes as potential drug targets for chronic pain because the expression of most family members (18 in humans and 50 in mice) is limited to nociceptors (Bender et al., 2002; Dong et al., 2001; Lembo et al., 2002). Furthermore, unlike most other ion channels involved in pain transduction, there is not widespread expression of these signaling proteins throughout the body, thus opening the door to blocking these genes specifically in nociceptors without perturbing non-nociceptive cellular functions. Thus far, the Mrgpr family of genes have been found in nociceptive primary afferents that innervate the skin, lung, colon, and tooth pulp (Bautzova et al., 2018; Chung et al., 2012; Han et al., 2018; Zylka et al., 2005). In terms of trigeminal ganglion expression of this receptor, it remains unclear which orofacial peripheral tissues the MRGPRX1+ afferents innervate. I will henceforth refer to this gene as hMrgprX1, as is conventionally done to distinguish the human from the rodent gene. Previous studies have shown that activating the mouse homologue of hMRGPRX1 blocks persistent inflammatory and neuropathic pain (Guan et al., 2010). In mice, when inflammation was triggered with hind paw injection of Complete Freund’s Adjuvant, intrathecal injection of an agonist to activate the mouse homologue of hMRGPRX1, greatly reduced the thermal hyperalgesia experienced during inflammatory pain (Guan et al., 2010). These analgesic results were validated in the mouse using elegant genetic targeting where the mouse homologue of the gene was replaced with hMrgprX1 (Li et al., 2017). Furthermore, the painkilling mechanism of action for hMRGPRX1 appears to involve a suppression of neurotransmitter release from nociceptors (Li et al., 2017). In summary, these findings highlight the analgesic action of this nociceptive-specific receptor, and suggests that directly activating this receptor may be sufficient to blunt unrelenting pain that is driven by local inflammation. The P2X receptor is another family that has garnered much attention in recent years due to accumulating evidence of its involvement in inflammatory and persistent pain, specifically its direct involvement in central sensitization causing hyperalgesia and allodynia (Burnstock 2000; Chen et al. 2014; Cherkas et al. 2012). P2X are ligand-gated ion channels that are activated by extracellular ATP (Cook et al. 1997). ATP is well recognized as an energy source and a modulator of cellular function. In the nervous system, ATP acts as a neurotransmitter and serves as a mediator of pain through binding to P2X receptor. ATP can be released actively or passively by cell lysis during tissue damage, which in turn may activate P2X receptors to initiate nociceptive signals. This effect may be exaggerated under conditions of inflammation. Many previous studies showed the expression of P2X in the central and peripheral neurons (Chen et al. 2014; Dunn et al. 2001; Bo et al. 1999; Llewellyn et al. 1998). In the orofacial region, it has been reported that P2X immunoreactivity exists in dental pulp, taste bud, and temporomandibular joint of the rat (Cook et al. 1997; Bo et al. 1999; Ichikawa et al. 2004). The presence of P2X in the human dental pulp has also been documented (Alavi et al. 2001; Renton et al. 2003). The presence of P2X in fibers of human dental pulp suggests that they may play a role in the perception of dental pain. Experimental dentin stimulation in human teeth produces ATP, which is detected by purinergic receptors by dental pulp afferents (Liu et al., 2015). Lipopolysaccharide, an antigen from infecting bacteria, increases the expression of P2X2, P2X3, and P2X5 when applied directly to the tooth pulp of rats (Chen et al., 2014). Application of a P2X1-3 receptor agonist, meATP, to exposed tooth pulp elicits a significant EMG response in anesthetized rats, and produces changes in the central nervous system associated with pain (Adachi et al., 2010). Furthermore, these responses could be blocked by pulp application of P2X1-3 antagonist TNP-ATP (Cherkas et al. 2012). Similar findings were found with P2X7 as well (Itoh et al. 2011). In summary, these findings strongly suggest a role for this receptor family in the transmission of dental pain.

**Study Design**

**Phase**
Not applicable

**Design**

Extracted teeth will be collected from a study participant and immediately frozen until subsequent experiments. To determine if hMRGPRX1 and P2X are expressed in human tooth pulp, we will use commercially available antisera directed against hMRGPRX1 and P2X, and perform serial section immunostaining on tooth pulp surgically removed from teeth. 20 teeth will be collected from each group (Group 1: normal pulp with normal periodontium; Group 2: asymptomatic irreversible pulpitis; Group 3: symptomatic irreversible pulpitis; Group 4: normal pulp with periodontal involvement) and all histological imaging will take place using SPE Leica Confocal microscope. All immunostaining experiments on control and experimental groups will be performed in parallel, with the experimenter analyzing and scoring the data blinded to which group each image comes from. 10 sections each from each group will be counterstained with antisera against (1) PGP9.5 to identify nerve fibers, where expression of this marker should be consistent across samples; and (2) TLR-4 and (3) CD14 to visualize inflammation of the pulp and its colocalization with hMRGPRX1 and/or P2X. Other markers may include but are not limited to CGRP, Substance P, TNF-alpha, TRPV1, TRPA1. We will quantify relative expression levels of marker levels using NIH Image J software, followed by students t-test to determine any statistical significance in expression levels across the groups. Colocalization and correlation analysis will be performed to study any relationship between markers.

**Study duration**

We anticipate it will take approximately 48 months to collect 80 extracted teeth from study participants and complete all analysis of the samples. The proposed dates of the project are October 2020 - June 2023. Each individual participant's length of participation will take only around 10-15 minutes prior to the extraction (time included to perform routine diagnostic tests and fill out a questionnaire form based in Redcap).

**Resources necessary for human research protection**

Describe research staff and justify that the staff are adequate in number and qualifications to conduct the research. Describe how you will ensure that all staff assisting with the research are adequately informed about the protocol and their research related duties. Please allow adequate time for the researchers to conduct and complete the research. Please confirm that there are adequate facilities for the research.

The research staff will include members of Dr. Claire Mitchell and Dr. Ishmail Abdus-Saboor's labs. All staff members have completed the necessary training to handle basic science research. Regular meetings (ranging from a weekly to monthly basis) have been held to discuss details on the research project, with all members being present and well informed of the protocol and their research duties in contribution to the project. Between the two labs and also collaboration with oral surgery faculty, Dr. Steven Wang and Dr. Helen Giannakopulos, there are more than adequate facilities to conduct this research.

**Characteristics of the Study Population**

**Target population**

For the pulpitis groups, the target population is adult patients (over the age of 18 years) presenting to Oral Surgery Clinic of the School of Dental Medicine for tooth extraction. For the control teeth, the target population will include younger patients (over the age of 12) presenting to Oral Surgery Clinic of the School of Dental Medicine for tooth extraction.
Subjects enrolled by Penn Researchers
80

Subjects enrolled by Collaborating Researchers
0

Accrual
We will need approximately 80 samples of normally discarded extracted teeth and questionnaires. The collection will be done in a de-identified fashion. The patient will be assigned a number on his or her consent form. This number will be written on the vial in which the extracted tooth is placed and on the questionnaire. The only record of participation in the study will be the consent forms. These will be maintained by the principal investigator. The individuals processing the tissue and questionnaire information will not know the name of the donor and will not be able to associate the number with the donors identity.

Key inclusion criteria
For the normal/control group, the inclusion criteria is patients requiring extraction over the age of 12. No one will be excluded based on gender, race, or ethnicity. For the experimental pulps groups, the inclusion criteria is patients requiring extraction over the age of 18. Likewise, no one will be excluded based on gender, race, or ethnicity.

Key exclusion criteria
For the control group, the exclusion criteria is patients under the age of 12 years. For the pulps groups, the exclusion criteria is patients under the age of 18 years and patients whose teeth do not demonstrate signs/symptoms of irreversible pulps.

Vulnerable Populations

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<thead>
<tr>
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<th>Children Form</th>
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<tr>
<td>Pregnant women (if the study procedures may affect the condition of the pregnant woman or fetus) Form</td>
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<td>Fetuses and/or Neonates Form</td>
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<td>Prisoners Form</td>
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<td>Other</td>
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None of the above populations are included in the research study

The following documents are currently attached to this item:

There are no documents attached for this item.

Populations vulnerable to undue influence or coercion
Although not directly targeted, mentally disabled persons, economically or educationally disadvantaged persons, and/or employees or students of the University of Pennsylvania will not be denied enrollment and any special protections and/or additional safeguards will be undertaken in order to protect the rights and welfare of these subjects from coercion or undue influence as appropriate.

Subject recruitment
The samples we propose to collect are extracted teeth that are removed at Oral Surgery Clinic of the Penn School of Dental Medicine. When samples are required, one of the investigators will attend the Oral Surgery Clinic and ask patients if they are willing to allow us to use their extracted tooth for research purposes, undergo a diagnostic examination, and fill out a short questionnaire. Informed consent will be obtained. Typically, several extractions are done in a clinic session so there is not a need for active recruitment of specimen donors.

Will the recruitment plan propose to use any Penn media services (communications, marketing, etc.) for outreach via social media avenues (examples include: Facebook, Twitter, blogging, texting, etc.) or does the study team plan to directly use social media to recruit for the research?
No
The following documents are currently attached to this item:

There are no documents attached for this item.

**Subject compensation***
Will subjects be financially compensated for their participation?
No

The following documents are currently attached to this item:

There are no documents attached for this item.

If there is subject compensation, provide the schedule for compensation per study visit or session and total amount for entire participation, either as text or separate document

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**Study Procedures**

**Suicidal Ideation and Behavior**
Does this research qualify as a clinical investigation that will utilize a test article (ie- drug or biological) which may carry a potential for central nervous system (CNS) effect(s)?
No

**Procedures**
As described above, extracted teeth will be immediately transported to the laboratory to be frozen until further experimentation. Serial section immunostaining will be performed on the tooth pulp using antisera directed against nociceptive and inflammatory genes (i.e. hMRGPRX1 and P2X). If these genes are present, comparisons between normal and inflamed teeth will be made, along with evaluation of the relationship of other factors that will be taken from the questionnaire (ie. pain to mastication, pain to cold, pain intensity, relation to migraines). The patient's dental records at Penn Dental will not be reviewed. The only records that will be under review are the ones obtained from the routine examination and questionnaire that was done immediately prior to the extraction.

The following documents are currently attached to this item:

There are no documents attached for this item.

**Deception**
Does your project use deception?
No

**International Research**
Are you conducting research outside of the United States?
No

**Analysis Plan**
Relative expression levels of hMRGPRX1, P2X, PGP9.5, TLR4, CD14 and more will be quantified using NIH Image J software, followed by student's t-test to determine any statistical significance in expression levels across the groups. Colocalization and correlation analysis will be performed to study the relationship between the different markers.

The following documents are currently attached to this item:

There are no documents attached for this item.
**Data confidentiality**

- Paper-based records will be kept in a secure location and only be accessible to personnel involved in the study.
- Computer-based files will only be made available to personnel involved in the study through the use of access privileges and passwords.

Prior to access to any study-related information, personnel will be required to sign statements agreeing to protect the security and confidentiality of identifiable information.

Wherever feasible, identifiers will be removed from study-related information.

A Certificate of Confidentiality will be obtained, because the research could place the subject at risk of criminal or civil liability or cause damage to the subject's financial standing, employability, or liability.

A waiver of documentation of consent is being requested, because the only link between the subject and the study would be the consent document and the primary risk is a breach of confidentiality. (This is not an option for FDA-regulated research.)

Precautions are in place to ensure the data is secure by using passwords and encryption, because the research involves web-based surveys.

Audio and/or video recordings will be transcribed and then destroyed to eliminate audible identification of subjects.

**Subject Confidentiality**

All questionnaires and extracted teeth will be collected in a deidentified fashion. The questionnaire will be given a number and once the tooth is removed from the patient's mouth and placed in a collection tube, the tube will be given a number. There will be no link between identifiers (e.g. name on the consent) and the subject's ID code. The samples will be transported to the laboratory for processing.

The only record of participation in the study will be the consent forms. The consent forms will be kept in a locked cabinet in Dr. Mitchell's office under her direct supervision. The questionnaires will all be based in Redcap, and only accessible to Dr. Mitchell and Lily See. The individuals processing the tissue will not know the name of the donor and will not be able to associate the number with the donor's identity. Furthermore, the team members from Penn Medicine will not be given any access to any patient PMI existing on the Penn Dental network server.

**Sensitive Research Information**

Does this research involve collection of sensitive information about the subjects that should be excluded from the electronic medical record?

No

**Subject Privacy**

Privacy refers to the person's desire to control access of others to themselves. Privacy concerns people, whereas confidentiality concerns data. Describe the strategies to protect privacy giving consideration to the following: The degree to which privacy can be expected in the proposed research and the safeguards that will be put into place to respect those boundaries. The methods used to identify and contact potential participants. The settings in which an individual will be interacting with an investigator. The privacy guidelines developed by relevant professions, professional associations and scholarly disciplines (e.g., psychiatry, genetic counseling, oral history, anthropology, psychology).

To protect patient privacy, the study will be discussed and consent obtained in a private room.

**Data Disclosure**

Will the data be disclosed to anyone who is not listed under Personnel?

Not Applicable
Data Protection*

- Name
  - Street address, city, county, precinct, zip code, and equivalent geocodes
- All elements of dates (except year) for dates directly related to an individual and all ages over 89
  - Telephone and fax number
  - Electronic mail addresses
  - Social security numbers
  - Medical record numbers
  - Health plan ID numbers
  - Account numbers
  - Certificate/license numbers
  - Vehicle identifiers and serial numbers, including license plate numbers
  - Device identifiers/serial numbers
  - Web addresses (URLs)
  - Internet IP addresses
  - Biometric identifiers, incl. finger and voice prints
  - Full face photographic images and any comparable images
  - Any other unique identifying number, characteristic, or code

None

Does your research request both a waiver of HIPAA authorization for collection of patient information and involve providing Protected Health Information ("PHI") that is classified as a "limited data set" (city/town/state/zip code, dates except year, ages less than 90 or aggregate report for over 90) to a recipient outside of the University of Pennsylvania covered entity?

No

Tissue Specimens Obtained as Part of Research*

Are Tissue Specimens being obtained for research?
Yes

Tissue Specimens - Collected during regular care*

Will tissue specimens be collected during regulator clinical care (for treatment or diagnosis)?
Yes

Tissue Specimens - otherwise discarded*

Would specimens otherwise be discarded?
Yes

Tissue Specimens - publicly available*

Will tissue specimens be publicly available?
No

Tissue Specimens - Collected as part of research protocol*

Will tissue specimens be collected as part of the research protocol?
Yes

Tissue Specimens - Banking of blood, tissue etc. for future use*

Does research involve banking of blood, tissue, etc. for future use?
Yes

Genetic testing

If genetic testing is involved, describe the nature of the tests, including if the testing is predicative or exploratory in nature. If predictive, please describe plan for disclosing results to subjects and provision
of genetic counseling. Describe how subject confidentiality will be protected Note: If no genetic testing is to be obtained, write: "Not applicable."

The extracted tooth will only be used to evaluate expression of suspected nociceptive and inflammatory genes. This testing is exploratory in nature, to determine if they are upregulated in the symptomatically inflamed pulp. The results will not be disclosed to the participants and no other genetic testing will be conducted.

## Consent

### 1. Consent Process

**Overview**

A verbal discussion with the potential tooth donor will take place to briefly explain the research study and determine if the person is willing and interested in participation. If interest/willingness is shown, one of the investigators will ask potential tooth donors to read the consent form prior to the initiation of their extraction. All of the subjects concerns and/or questions will be addressed until they are satisfied. We will emphasize the points that: 1) their decision will not affect their care and 2) the teeth and questionnaires will be collected in a manner such that it will be impossible for the investigators to determine the identity of the tooth donor. At that point they will be asked to sign the consent form if they are willing to donate their tooth and participate in the study.

**Children and Adolescents**

An overview of the research, procedures we would perform, and the potential involvement of the child will be clearly explained to both the child/adolescent and their parent/guardian. The parent/guardian will be asked to read the consent form prior to the initiation of their child's extraction. The child will be asked to read the assent form. All concerns and/or questions will be addressed until they are satisfied. We will emphasize the points that: 1) their decision will not affect their care and 2) the teeth and questionnaires will be collected in a manner such that it will be impossible for the investigators to determine the identity of the tooth donor. If both the child/adolescent and parent/guardian agree to participation in the study, the parent/guardian will be asked to sign the consent form.

**Adult Subjects Not Competent to Give Consent**

Not Applicable

### 2. Waiver of Consent

**Waiver or Alteration of Informed Consent**

- No Waiver Requested

**Minimal Risk**

**Impact on Subject Rights and Welfare**

**Waiver Essential to Research**

**Additional Information to Subjects**

**Written Statement of Research**

- No

If no written statement will be provided, please provide justification

The following documents are currently attached to this item:

*There are no documents attached for this item.*
Risk / Benefit

Potential Study Risks
For the possible risk of breach of confidentiality, every effort will be made to ensure participants' privacy is maintained. During the consent process, consent will be obtained in a private room. The principle investigator will ensure consent forms are maintained in a locked filing cabinet that only she can access. For the possible risks of genetic testing, only inflammatory and nociceptive gene expression (i.e. P2X, CGRP, TNF-alpha) will be examined. These genes cannot identify the individual. No other genetic testing will be conducted. The samples will be deidentified and the results will not be shared with study participants.

Potential Study Benefits
There are no direct benefits to patients who agree to donate their teeth and time. It is our hope that the data generated during these studies will enhance our understanding of chronic dental pain and lead to the development of novel therapeutic modalities.

Alternatives to Participation (optional)

Data and Safety Monitoring
Not Applicable

The following documents are currently attached to this item:

*There are no documents attached for this item.*

Risk / Benefit Assessment
Participation in this research study comes at no added risk than the standard of care to the patient, as we will only be performing routine diagnostic tests and having them answer a short questionnaire. The donated tooth is one that they have already planned to extract as well. Likewise, the participants are unlikely to have any added benefit from participation. Potential benefits from this research, however, include a better understanding of what causes dental pain, factors associated with dental pain, and potentially how to prevent and treat it more effectively in the future.

General Attachments

*The following documents are currently attached to this item:*

- Questionnaires (questionnaire.pdf)
- Additional forms (assentform.doc)
- Supplemental form(s) (childform.doc)
- Informed consent form (833399consentform_2020.docx)
UNIVERSITY OF PENNSYLVANIA
RESEARCH SUBJECT
INFORMED CONSENT/ PARENTAL PERMISSION AND HIPPA AUTHORIZATION FORM

Protocol Title: Characterization of Pain Receptor Expression in the Human Dental Pulp

Principal Investigator: Claire Mitchell, PhD. Professor.
Levy Building, Room 431
240S 40th St
Philadelphia, PA 19104
(215) 573-2176
Email: chm@dental.upenn.edu

Emergency Contact: Claire Mitchell, PhD. Professor
(215) 573-2176

This consent form is written from the point of view of a research participant. If the parent or legal guardian of a minor or a legally authorized representative will be providing consent, the words "you" and "your" should be read as ("your child" or "the research participant").

Summary of Key Information
You are being invited to participate in a research study. Your participation is voluntary and you should only participate if you completely understand what the study requires and what the risks of participation are. You should ask the study team any questions you have related to participating before agreeing to join the study. If you have any questions about your rights as a human research participant at any time before, during or after participation, please contact the Institutional Review Board (IRB) at (215) 898-2614 for assistance.

The research study is being conducted to study the role of a pain receptor in the dental pulp. You are eligible for this study as you are about to get an extraction: whether it be for orthodontic or restorability purposes.

If you agree to join the study, you will be asked to complete the following research procedures: a diagnostic examination of the tooth and questionnaire prior to the extraction of your tooth. We would also keep the tooth for further research studies in the future.

Your participation will last for 5-10 minutes. After which, you will proceed with the tooth extraction.
You are not expected to gain any benefits from your participation in this research study. Also, there are no risks to your participation in this study.

Please note that there are other factors to consider before agreeing to participate such as additional procedures, use of your personal information, costs, and other possible risks not discussed here. If you are interested in participating, a member of the study team will review the full information with you. You are free to decline or stop participation at any time during or after the initial consenting process.

**Why am I being asked to volunteer?**
You are being invited to participate in a research study because you are going to have a tooth extraction to address a problem with your teeth.

Your doctor may be an investigator in this research study. You do not have to participate in any research study offered by your doctor. If you choose not to participate, there will be no loss of benefits to which you are otherwise entitled. You may also decide to discuss the study with your family, friends, or family doctor. Being in a research study is different from being a patient. As an investigator, your doctor is interested both in your clinical welfare and in the conduct of this study.

If you decide to participate, you will be asked to sign this form.

**What is the purpose of this research study?**
The purpose of this study is to increase understanding of pain receptors in dental pulp through the examination of certain gene receptors. We will compare healthy and symptomatic dental pulp for this purpose. We will assess whether this is related to other pain factors such as stimulus, migraines, etc.

**How long will I be in the study/ How many other people will be in the study?**
The additional required research procedures will take approximately 5-10 minutes before the procedure begins. The research will not have an effect on your care.

We are aiming to enroll approximately 80 patients at the University Of Pennsylvania School of Dental Medicine.

**What am I being asked to do?**
You are going to have a tooth extraction. Ordinarily this tooth would be thrown in the trash. We would like to keep it for use in future research studies. If you agree, this tooth will be kept and used in research.

Before the extraction we would also like to perform some diagnostic tests on the tooth and have you to answer a short questionnaire. The questionnaire will be used to study the quality of pain and other factors that may predispose people to pain.

The routine diagnostic tests on your tooth and 2 nearby teeth include percussion (tapping the tooth), palpation (putting finger pressure on your gums), and thermal testing (ie. applying a cold cotton pellet to the tooth for 2-3 seconds). Following extraction, the
extracted tooth will be placed in a vial and taken to the laboratory for future study of
pain.

What are the possible risks or discomforts?
The diagnostic examination and questionnaire will in no way increase the risk of the
procedure you will be undergoing.

Your extracted tooth will undergo molecular testing for genes involved in pain. Your
samples will be de-identified. The researchers working with the samples will not be able
to connect you with the samples you have donated.

With most research there is a risk of breach of confidentiality of your data. However, the
tooth sample, diagnostic information, and questionnaire will be collected in a de-
identified fashion.

Risks of Genetic Testing
This research includes genetic testing. Even without your name or other identifiers, your
genetic information is unique to you. The researchers believe the chance that someone
will identify you is very small, but the risk may change in the future as people come up
with new ways of tracing information.

What if new information becomes available about the study?
During the course of this study, we may find more information that could be important to
you. This includes information that, once learned, might cause you to change your mind
about being in the study. We will notify you as soon as possible if such information
becomes available.

What are the possible benefits of the study?
You are not expected to gain any benefits by donating your extracted tooth. The benefits
of research using your tooth include learning more about what causes dental pain, how to
prevent it, what factors it is associated with, and how to treat it more effectively in the
future.

What other choices do I have if I do not participate?
The choice to fill out the questionnaire and let us perform routine diagnostic tests and
keep the discarded tooth for research is entirely up to you. No matter what you decide to
do, it will not affect your care.

Will I be paid for being in this study?
You will not receive financial compensation for donating your extracted tooth.

Will I have to pay for anything?
You will not have pay for participating in the study. However, you (or your insurance
carrier) are still responsible for the cost of your tooth extraction.

Will I receive the results of research testing?
Most tests done in research studies are only for research and have no clear meaning for health care. Research results will not be returned to you because they would not be relevant to your health care.

Who can see or use my information? How will my personal information be protected?
We will do our best to make sure that the personal information obtained during the course of this research study will be kept private. However, we cannot guarantee total privacy. Your personal information may be given out if required by law. If information from this study is published or presented at scientific meetings, your name and other personal information will not be used. The Institutional Review Board (IRB) at the University of Pennsylvania will have access to your records. If this study is being overseen by the Food and Drug Administration (FDA), they may review your research records.

To protect your identity, signed consent forms will be secured in a locked filling cabinet in the principle investigator’s office. Your information/questionnaire/specimens will be assigned as unique random study ID to de-identify your name from your samples.

What may happen to my information and samples collected on this study?
Your samples may be used to create products, including some that may be sold and/or make money for others. If this happens, there are no plans to tell you, or to pay you, or to give any compensation to you or your family.

Whole genome sequencing will not be conducted on your teeth samples. Whole genome sequencing involves analyzing your entire personal genetic code.

Your information and samples could be stored and shared for future research in a de-identified fashion. De-identified means that all identifiers will have been removed prior to sharing. It would not be possible for future researchers to identify you as we would not share any identifiable information about you with future researchers. This can be done without again seeking your consent in the future, as permitted by law. The future use of your information and samples only applies to the information and samples collected on this study.

What information about me may be collected, used or shared with others?
- Name, elements of dates
- Personal dental history via the questionnaire

Why is my information being used?
Your information is used by the research team to contact you during the study. Your information and results of tests and procedures are used to:
- do the research
- oversee the research
- to see if the research was done right
- to evaluate and manage research functions.
Who may use and share information about me?
The following individuals may use or share your information for this research study:
- The investigator for the study and the study team
- Other authorized personnel at Penn, including offices that support research operations
- Other research personnel with access to the databases for research and/or study coordination and as otherwise approved by the IRB

Who, outside of Penn Dental, might receive my information?
Oversight organizations
- The U. S. Office of Human Research Protections (OHRP)

Once your personal health information is disclosed to others outside Penn Dental, it may no longer be covered by federal privacy protection regulations.

The Principal Investigator or study staff will inform you if there are any additions to the list above during your active participation in the trial. Any additions will be subject to University of Pennsylvania procedures developed to protect your privacy.

How long may Penn Dental use or disclose my personal health information?
Your authorization for use of your personal health information for this specific study does not expire.

Your information may be held in a research database. However, Penn Dental may not re-use or re-disclose information collected in this study for a purpose other than this study unless:
- You have given written authorization
- The University of Pennsylvania’s Institutional Review Board grants permission
- As permitted by law

Can I change my mind about giving permission for use of my information?
Yes. You may withdraw or take away your permission to use and disclose your health information at any time. You do this by sending written notice to the investigator for the study. If you withdraw your permission, you will not be able to stay in this study.

What if I decide not to give permission to use and give out my health information?
Then you will not be able to be in this research study. You will be given a copy of this Research Subject HIPAA Authorization describing your confidentiality and privacy rights for this study. By signing this document, you are permitting Penn Dental to use and disclose personal health information collected about you for research purposes as described above.
Who can I call with questions, complaints or if I’m concerned about my rights as a research subject?
If you have questions, concerns or complaints regarding your participation in this research study or if you have any questions about your rights as a research subject, you should speak with the Principal Investigator listed on page one of this form. If a member of the research team cannot be reached or you want to talk to someone other than those working on the study, you may contact the Office of Regulatory Affairs with any question, concerns or complaints at the University of Pennsylvania by calling (215) 898-2614.
When you sign this form, you are agreeing to take part in this research study. This means that you have read the consent form, your questions have been answered, and you have decided to volunteer. Your signature also means that you are permitting the University of Pennsylvania to use your personal health information collected about you for research purposes within our institution. You are also allowing the University of Pennsylvania to disclose that personal health information to outside organizations or people involved with the operations of this study.

A copy of this consent form will be given to you.

________________________
Subject’s Printed Name

If subject is 18 years of age and older:

________________________       ___________________
Subject’s Signature       Date

If subject is younger than 18 years of age:

________________________       ________________________       _____________
Parent/Legal Guardian's       Parent/Legal Guardian's       Date
Printed Name       Signature

Relation: □ Mother □ Father □ Legal Guardian

________________________       _____________________________ ___________
Name of Person Obtaining  Signature       Date
Consent (Please Print)
ASSENT TO PARTICIPATE IN RESEARCH

1. My name is

2. We are asking you to take part in a research study because we are trying to learn more about

If you agree to be in this study

6. Please talk this over with your parents before you decide whether or not to participate. We will also ask your parents to give their permission for you to take part in this study. But even if both of your parents say “yes” you can still decide not to be in this study.

7. If you don’t want to be in this study, you do not have to participate. Remember, being in this study is up to you and no one will be upset if you don’t want to participate or even if you change your mind later and want to stop.

8. You can ask any questions that you have about this study. If you have a question later that you didn’t think of now, you can call me or ask me the next time you see me.

9. Signing your name below means that you agree to be in this study. You and your parents will be given a copy of this form after you sign it.

_________________________________________  ____________________
Participant                                   Date

_________________________________________  ____________________
Investigator                                Date
<table>
<thead>
<tr>
<th>#</th>
<th>Variable / Field Name</th>
<th>Field Label</th>
<th>Field Attributes (Field Type, Validation, Choices, Calculations, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>subject_id</td>
<td>Subject ID</td>
<td>text</td>
</tr>
</tbody>
</table>
| 2  | vitals_taken          | 1. Were vital signs collected? | yes/no, Required
1. Yes
0. No |
| 3  | date_taken            | 2. On what date were the measurements performed? | text (date_mdy) Custom alignment: RH |
| 4  | time_taken            | 3. At what time were the measurements performed? | text (time) Custom alignment: RH |
| 5  | temp                  | 4. Oral Temperature (°C) enter temperature (°C) | text (number_1dp, Min: 36.0, Max: 39.0) Custom alignment: RH |
| 6  | systolic_bp           | Systolic Blood Pressure (mmHg) | text (integer) Custom alignment: RH |
| 7  | diastolic_bp          | Diastolic Blood Pressure (mmHg) | text (integer) Custom alignment: RH |
| 8  | bp_location           | 5. BP Location | radio
1. Left Arm
2. Right Arm |
| 9  | pulse                 | 6. Pulse Rate beats per minute | text (integer) Custom alignment: RH |
| 10 | comments              | Comments | notes Custom alignment: LV |
| 11 | vitals_complete       | Section Header: Form Status Complete? | dropdown
0. Incomplete
1. Unverified
2. Complete |
### Instrument: Informed Consent Documentation

<table>
<thead>
<tr>
<th>Field</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>subject_initials</td>
<td>1. Subject Initials text (alpha only)</td>
</tr>
<tr>
<td>icf_date</td>
<td>2. Informed Consent Date text (date_mdy), Required</td>
</tr>
<tr>
<td>icf_time</td>
<td>3. Time of Consent text (time)</td>
</tr>
<tr>
<td>icf</td>
<td>4. Informed Consent Documentation checkbox, Required</td>
</tr>
<tr>
<td>icf_comments</td>
<td>5. Comments: notes</td>
</tr>
<tr>
<td>informed_consent_documentation_complete</td>
<td>Section Header: Form Status Complete?</td>
</tr>
</tbody>
</table>

#### icf

- **icf_1**: The subject was given sufficient time to review the consent form and to ask questions.
- **icf_2**: Site personnel answered all of the subject's questions about participation in the study.
- **icf_3**: Interventionist participated in the informed consent process.
- **icf_4**: Consent was obtained prior to any study procedures being performed.
- **icf_5**: A copy of the signed and dated consent form was given to the subject.
- **icf_6**: Please check the box if ICF was presented orally to the subject and document in the note below.

### Instrument: Demographics

<table>
<thead>
<tr>
<th>Field</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>initials</td>
<td>1. Subject Initials text, Required</td>
</tr>
<tr>
<td>dob</td>
<td>2. What is the subject's Date of Birth? (Record the date of birth using the MM-DD-YYYY format.) text (date_mdy), Required, Identifier</td>
</tr>
<tr>
<td>subject_age</td>
<td>3. What is the subject's age? calc, Required, Identifier Calculation: round(datediff([dob], [date_demo],'y', 'mdy', 'false'), 1) Custom alignment: RH</td>
</tr>
<tr>
<td>date_demo</td>
<td>4. What is the date of collection? (Record the date of demographics collection) text (date_mdy), Identifier</td>
</tr>
<tr>
<td>bio_sex</td>
<td>5. What is the biological sex of the subject? (Record the appropriate sex. Collect the sex, as reported by the subject or caretaker. Select one.) radio, Required</td>
</tr>
<tr>
<td>ethnicity</td>
<td>6. What is the ethnicity of the subject? (Study participants should self-report ethnicity, with ethnicity being asked about before race. Select one.) radio, Required</td>
</tr>
</tbody>
</table>
### Section Header: Form Status

<table>
<thead>
<tr>
<th>Complete?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 incomplete</td>
</tr>
<tr>
<td>1 Unverified</td>
</tr>
<tr>
<td>2 Complete</td>
</tr>
</tbody>
</table>

### Instrument: Medications (medications)

<table>
<thead>
<tr>
<th>Field</th>
<th>Question</th>
<th>Type</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>med_taken</td>
<td>1. Were any medications taken?</td>
<td>yes/no</td>
<td>1 Yes</td>
</tr>
<tr>
<td>date_collect</td>
<td>2. What is the date of collection?</td>
<td>text (date_mdy)</td>
<td></td>
</tr>
<tr>
<td>med_name</td>
<td>3. What was the name of the medication?</td>
<td>text</td>
<td>BIOPORTAL:RXNORM BIOPORTAL:RXNORM</td>
</tr>
<tr>
<td>med_class</td>
<td>4. Under what classification does this medication fall?</td>
<td>radio</td>
<td>1 Pain</td>
</tr>
<tr>
<td>other_med</td>
<td>If other, please explain:</td>
<td>text</td>
<td></td>
</tr>
<tr>
<td>pain_med_purpose</td>
<td>Purpose of pain medication</td>
<td>radio</td>
<td>1 Dental Pain</td>
</tr>
<tr>
<td>dose_medic</td>
<td>5. What was the individual dose of the medication?</td>
<td>text</td>
<td>5 What was the individual dose of the medication?</td>
</tr>
</tbody>
</table>

### Race

7. What is the race of the subject? (Study participants should self-report race, with race being asked about after ethnicity. Check all that apply.)

#### Options
- 1 race__1 Black/African American
- 2 race__2 American Indian/Alaska Native
- 3 race__3 Asian
- 4 race__4 Native Hawaiian/Other Pacific Islander
- 5 race__5 White/Caucasian
- 6 race__6 Unknown
- 7 race__7 Not reported
### dose_unit
Show the field ONLY if:  
[med_taken] = '1'

#### 6. What was the dose unit of the medication/therapy?

<table>
<thead>
<tr>
<th>radio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mg</td>
</tr>
<tr>
<td>2</td>
<td>ug</td>
</tr>
<tr>
<td>3</td>
<td>mL</td>
</tr>
<tr>
<td>4</td>
<td>g</td>
</tr>
<tr>
<td>5</td>
<td>IU</td>
</tr>
<tr>
<td>6</td>
<td>ng/kg/min</td>
</tr>
<tr>
<td>7</td>
<td>L/min</td>
</tr>
<tr>
<td>8</td>
<td>mg/mL</td>
</tr>
<tr>
<td>9</td>
<td>Teaspoon</td>
</tr>
<tr>
<td>10</td>
<td>Tablespoon</td>
</tr>
</tbody>
</table>

### dose_form
Show the field ONLY if:  
[med_taken] = '1'

#### 7. What was the dosage form of the medication/therapy?

<table>
<thead>
<tr>
<th>radio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aerosol</td>
</tr>
<tr>
<td>2</td>
<td>Capsule</td>
</tr>
<tr>
<td>3</td>
<td>Cream</td>
</tr>
<tr>
<td>4</td>
<td>Gas</td>
</tr>
<tr>
<td>5</td>
<td>Gel</td>
</tr>
<tr>
<td>6</td>
<td>Ointment</td>
</tr>
<tr>
<td>7</td>
<td>Patch</td>
</tr>
<tr>
<td>8</td>
<td>Powder</td>
</tr>
<tr>
<td>9</td>
<td>Spray</td>
</tr>
<tr>
<td>10</td>
<td>Suppository</td>
</tr>
<tr>
<td>11</td>
<td>Suspension</td>
</tr>
<tr>
<td>12</td>
<td>Tablet</td>
</tr>
<tr>
<td>13</td>
<td>Injection</td>
</tr>
<tr>
<td>14</td>
<td>Infusion</td>
</tr>
<tr>
<td>15</td>
<td>Implant</td>
</tr>
<tr>
<td>16</td>
<td>Liquid</td>
</tr>
<tr>
<td>17</td>
<td>Drops</td>
</tr>
<tr>
<td>18</td>
<td>Chewable Tablets</td>
</tr>
<tr>
<td>19</td>
<td>Oral Rinse</td>
</tr>
</tbody>
</table>

### med_freq
Show the field ONLY if:  
[med_taken] = '1'

#### 8. What was the frequency of the medication/therapy?

**LEGEND**
- QD= Every day
- BID= Twice a day
- TID= Three times a day
- QID= Four times a day
- QOD= Every other day
- QM= Every morning
- PRN = As needed

<table>
<thead>
<tr>
<th>radio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>QD</td>
</tr>
<tr>
<td>2</td>
<td>BID</td>
</tr>
<tr>
<td>3</td>
<td>TID</td>
</tr>
<tr>
<td>4</td>
<td>QID</td>
</tr>
<tr>
<td>5</td>
<td>QOD</td>
</tr>
<tr>
<td>6</td>
<td>QM</td>
</tr>
<tr>
<td>7</td>
<td>PRN</td>
</tr>
<tr>
<td>8</td>
<td>UNKNOWN</td>
</tr>
</tbody>
</table>
9. What was the route of administration?

- Oral
- Topical
- Subcutaneous
- Transdermal
- Intramuscular
- Respiratory (Inhalation)
- Intravascular
- Intraperitoneal
- Nasal
- Vaginal
- Rectal
- Intravenous
- Sublingual
- Buccal

10. Is the start date of the medication/therapy known?

- Yes
- No

11. What was the start date of the medication/therapy?

- Text (date, mdy)

a. Start Date Partial or Unknown?

- Partial Date
- Unknown

12. Is the medication/therapy still ongoing?

- Yes
- No

13. What was the end date of the medication/therapy?

- Text (date, mdy)

a. End Date Partial or Unknown?

- Partial Date
- Unknown

Section Header: Form Status

- Complete
- Incomplete
- Unverified
<table>
<thead>
<tr>
<th>Instrument: <strong>Patient Survey</strong> (patient_survey)</th>
<th>Enabled as survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>reason_pain</strong></td>
<td>Is the reason for your extraction today related to dental pain?</td>
</tr>
<tr>
<td>1 Yes</td>
<td>2 No</td>
</tr>
<tr>
<td><strong>pain_length</strong></td>
<td>How long have you had this dental pain?</td>
</tr>
<tr>
<td>1 Past 1-3 days</td>
<td>2 Past 1-2 weeks</td>
</tr>
<tr>
<td><strong>current_pain</strong></td>
<td>Please select the number that corresponds to the level of pain intensity you are experiencing in your mouth right now</td>
</tr>
<tr>
<td>slider (number)</td>
<td>Slider labels: 0, No Pain, 50, 100, Worst Imaginable Pain</td>
</tr>
<tr>
<td>Custom alignment: RH</td>
<td></td>
</tr>
<tr>
<td><strong>worst_pain</strong></td>
<td>Please select the number that corresponds to the level of pain intensity you are experiencing in your mouth at its worst</td>
</tr>
<tr>
<td>slider (number)</td>
<td>Slider labels: 0, No Pain, 50, 100, Worst Imaginable Pain</td>
</tr>
<tr>
<td>Custom alignment: RH</td>
<td></td>
</tr>
<tr>
<td><strong>charac_pain</strong></td>
<td>Which characteristic best describes your pain? (check all that apply)</td>
</tr>
<tr>
<td>checkbox</td>
<td>1 charac_pain___1 Sharp</td>
</tr>
<tr>
<td><strong>local_pain</strong></td>
<td>Which best describes your pain?</td>
</tr>
<tr>
<td>radio</td>
<td>1 Localized (one area)</td>
</tr>
<tr>
<td><strong>oral_cavity_picture</strong></td>
<td></td>
</tr>
<tr>
<td>descriptive</td>
<td></td>
</tr>
<tr>
<td><strong>pain_other_area</strong></td>
<td>Select the other areas that the pain is also found</td>
</tr>
<tr>
<td>checkbox</td>
<td>1 pain_other_area___1 Forehead</td>
</tr>
<tr>
<td><strong>freq_pain</strong></td>
<td>How frequently does the pain occur?</td>
</tr>
<tr>
<td>radio</td>
<td>1 Continuous</td>
</tr>
<tr>
<td><strong>pain_item</strong></td>
<td>What increases your pain? (check all that apply)</td>
</tr>
<tr>
<td>checkbox</td>
<td>1 pain_item___1 Cold food or drink</td>
</tr>
<tr>
<td>56</td>
<td>conditions</td>
</tr>
<tr>
<td>----</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>Show the field ONLY if: [reason_pain] = '1'</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>57</th>
<th>headache_activity</th>
<th>Has a headache limited your activities for a day or more in the last three months?</th>
<th>radio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Show the field ONLY if: [conditions(1)] = '1'</td>
<td></td>
<td>1 Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>58</th>
<th>headache_nausea</th>
<th>Are you nauseated or sick to your stomach when you have a headache?</th>
<th>radio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Show the field ONLY if: [conditions(1)] = '1'</td>
<td></td>
<td>1 Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>59</th>
<th>headache_light</th>
<th>Does light bother you when you have a headache?</th>
<th>radio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Show the field ONLY if: [conditions(1)] = '1'</td>
<td></td>
<td>1 Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>patient_survey_complete</th>
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<td>Complete?</td>
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</table>

<table>
<thead>
<tr>
<th>Instrument: Interventionist Exam (interventionist_exam)</th>
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</table>

<table>
<thead>
<tr>
<th>61</th>
<th>tooth_number</th>
<th>Tooth Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1-32</td>
<td>text (integer, Min: 1, Max: 32) Custom alignment: RH</td>
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</table>

<table>
<thead>
<tr>
<th>62</th>
<th>tooth_type</th>
<th>Tooth Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>radio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Control tooth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Extracted tooth</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>63</th>
<th>cold_test</th>
<th>Cold Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Based on patient reaction</td>
<td>radio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 +/-WNL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 +/-L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 ++/WNL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 ++/L</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>64</th>
<th>percussion</th>
<th>Percussion</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>radio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 ++</td>
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</table>

<table>
<thead>
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<th>palpation</th>
<th>Palpatation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>radio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 +</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>66</th>
<th>bone_support</th>
<th>Bone support levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>radio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 &gt;75%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 50-75%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 &lt; 50%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>67</th>
<th>rad_interp</th>
<th>Radiographic Interpretation/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Description of tooth ie. large caries, restoration, existing crown, periapical status</td>
</tr>
<tr>
<td></td>
<td></td>
<td>notes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Custom alignment: LV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>68</th>
<th>interventionist_exam_complete</th>
<th>Section Header: Form Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete?</td>
<td>Complete?</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>dropdown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Incomplete</td>
</tr>
<tr>
<td></td>
<td>1 Unverified</td>
</tr>
<tr>
<td></td>
<td>2 Complete</td>
</tr>
</tbody>
</table>
References


[54] Cho T, Chaban VV. Expression of P2X3 and TRPV1 receptors in primary sensory neurons from estrogen receptors-α and estrogen receptor-β knockout mice. Neuroreport 2012;23(9):530-534.


[61] Coimbra F, Coimbra A. Dental noxious input reaches the subnucleus caudalis of the trigeminal complex in the rat, as shown by c-fos expression upon thermal or mechanical stimulation. Neuroscience letters 1994;173(1-2):201-204.


[125] Holland GR, Robinson PP. The number and size of axons at the apex of the cat's canine tooth. Anat Rec 1983;205(2):215-222.


