CD73 Controls Extracellular Adenosine Generation in the Trigeminal Nociceptive Nerves

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Abstract

Purinergic signaling is involved in pain generation and modulation in the nociceptive sensory nervous system. Adenosine triphosphate (ATP) induces pain via activation of ionotropic P2X receptors while adenosine mediates analgesia via activation of metabotropic P1 receptors. These purinergic signaling are determined by ecto-nucleotidases that control ATP degradation and adenosine generation. Using enzymatic histochemistry, we detected ecto-AMPase activity in dental pulp, trigeminal ganglia (TG) neurons, and their nerve fibers. Using immunofluorescence staining, we confirmed the expression of ecto-5'-nucleotidase (CD73) in trigeminal nociceptive neurons and their axonal fibers, including the nociceptive nerve fibers projecting into the brainstem. In addition, we detected the existence of CD73 and ecto-AMPase activity in the nociceptive lamina of the trigeminal subnucleus caudalis (TSNC) in the brainstem. Furthermore, we demonstrated that incubation with specific anti-CD73 serum significantly reduced the ecto-AMPase activity in the nociceptive lamina in the brainstem. Our results indicate that CD73 might participate in nociceptive modulation by affecting extracellular adenosine generation in the trigeminal nociceptive pathway. Disruption of TG neuronal ecto-nucleotidase expression and axonal terminal localization under certain circumstances such as chronic inflammation, oxidant stress, local constriction, and injury in trigeminal nerves may contribute to the pathogenesis of orofacial neuropathic pain.

Keywords: neuropathic pain, antinociception, purinergic signaling, ecto-nucleotidases, trigeminal nerves, orofacial pain

Introduction

Trigeminal nociceptive neurons and their nerve fibers that innervate orofacial tissues receive noxious stimulation and then transduce, conduct, and transmit the pain signal to the secondary nociceptive neurons in the brainstem. Adenosine triphosphate (ATP) and its metabolites, including adenosine, are important pain mediators and modulators in the sensory nervous system (Hamilton and McMahon 2000; Burnstock 2009). Purinergic signaling is dictated by ATP release, nucleotide hydrolysis, and subsequent purinergic receptor activation and deactivation. Activations of different purinergic receptors induce various affections in nociception. For example, ATP usually activates P2X receptors and induces pain in peripheral nerves (Wirkner et al. 2007); P2X receptors also participate in the central sensitization in neuropathic pain (Fabbretti 2013), while adenosine usually generates analgesia via activation of P1 receptors (Sawynok and Liu 2003). Four subtypes of P1 receptors (i.e., A1R, A2AR, A2BR, and A3R) have been detected in the nociceptive neurons, glia cells, and immune cells in the nervous system (Sawynok 2015).

Endogenous adenosine-induced analgesia depends on the extracellular level of adenosine, which can be affected by adenosine monophosphate (AMP) hydrolysis, adenosine transport, and degradation (Zylka 2011). Even though manipulation of adenosine transport or degradation can induce antinociception (Schmidt et al. 2009), the endogenous adenosine level is

mainly controlled by extracellular AMP hydrolysis. Indeed, it has been shown that extracellular AMP hydrolysis provides the major source for endogenous adenosine in the nervous system that is essential to maintain a tonic activation of adenosine receptors in spinal cord nociceptive neurons (Sowa et al. 2010). Two types of ecto-nucleotidases (CD73 and prostatic acid phosphatase [PAP]) have been identified to be responsible for extracellular AMP hydrolysis in the spinal cord (Street et al. 2011). Knockout of CD73 and/or PAP reduced adenosine generation and enhanced nociception in animal models following inflammation and nerve injury (Sowa et al. 2010). These results indicate that the endogenous adenosine plays a crucial role in impeding the pain signal transduction and transmission in the nociceptive circuits. It also suggests that manipulating

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A supplemental appendix to this article is available online.

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extracellular AMP hydrolysis would provide an alternative mechanism to control the pain (Zylka 2011).

ATP is released following tissue injury, inflammation, and cellular or synaptic vesicle release (Fabbretti 2013). Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) are the dominant enzymes responsible for extracellular ATP/adenosine diphosphate (ADP) hydrolysis (Zimmermann and Braun 1996), which is essential to provide the substitute (AMP) for adenosine generation. Our previous work have shown that NTPDase2 is exclusively expressed in Swann cells and satellite cells that encapsulate the trigeminal ganglia (TG) neurons and their nerve fibers (Liu et al. 2012), while NTPDase3 is specifically expressed in TG nociceptive neurons and their axonal fibers (Liu et al. 2015). Thus, the trigeminal nociceptive nerves are well equipped with the machineries for ATP/ADP hydrolysis. To support the involvement of endogenous adenosine in modulation of dental orofacial pain, the existence of ecto-nucleotidases responsible for AMP hydrolysis in the trigeminal nerves has yet to be identified.

In this study, we determined the functional activities of ecto-AMPase in trigeminal nerves. We also identified the expression of CD73 in TG nociceptive neurons and in the nociceptive lamina of the trigeminal subnucleus caudalis (TSNC) in the brainstem. By controlling the generation of adenosine, the neuronal CD73 is expected to play a significant role in affecting the pain signal transduction, transmission, and modulation in the trigeminal nociceptive pathway.

Materials and Methods

Sample Preparation

Extracted third molars with healthy pulp were collected from patients (aged 18–24 y) at the Eastman Institute for Oral Health at University of Rochester with the approval of the institutional research subject review board. The animal experiment was approved by the University of Rochester Committee on Animal Recourses. For dental pulp, TG, brainstem, or spinal cord sample preparation and slice sectioning, see the online Appendix.

Immunofluorescence Staining

Prepared TG, nerve trunk, or brainstem sections were first incubated with blocking solution (1% bovine serum albumin [BSA], 1% normal goat serum, and 0.03% Triton X-100 in 1× phosphate-buffered saline [PBS]) for 2 h at 4°C and then incubated overnight with primary antibodies in incubation solution (1% BSA, 1% normal goat serum in 1× PBS). The primary antibodies used in this study include mouse anti-MAP2 (1:1,000; Sigma) and rabbit anti-mouse CD73 (rNU-9I₅, 1:500). CD73 antibody was kindly provided by Dr. J. Seigny at University Laval. The specificity of the antibody from the same lot (rNU-9I₅) had been previously characterized (Martin-Satue et al. 2009; Fausther et al. 2012; Liu et al. 2012). For negative control, preimmune serum was used to replace the primary antibody serum. For IB4 staining, FITC-conjugated

Isolectin B4 (1:400; Enzo) was included in the incubation solution. For more details, see the online Appendix.

Ecto-nucleotidase Histochemistry

To demonstrate functional ecto-AMPase activity in dental pulps, TGs, brainstems, or spinal cords, we used a lead phosphate method as previously described (Liu et al. 2015). For more details, see the online Appendix.

Anti-CD73 Serum Incubation and Quantification for Ecto-AMPase Activity Staining

To demonstrate that CD73 mediates the ecto-AMPase activity detected in the nociceptive pathway, we tested if preincubation with specific anti-CD73 serum reduces the ecto-AMPase activity. For more detail, see the online Appendix. The differences in staining intensity between the anti-CD73 serum-preincubated sections and the control serum-preincubated sections were analyzed using unpaired Student's *t* test. A total of 10 sections were used in each group (*n* = 10). The data were presented as means ± SE. *P* < 0.05 is considered as statistically significant difference.

Results

Detection of Functional Ecto-AMPase Activity in Dental Pulps and Trigeminal Nerves

Ecto-nucleotidase-mediated AMP hydrolysis provides the major source of extracellular adenosine (Street et al. 2011). We tested if ecto-AMPase activity is detectable in dental pulp and trigeminal nerves. As shown in Figure 1A, ecto-AMPase activity was detected in human dental pulp as illustrated by positive brown deposits in odontoblast layer, subodontoblast region, and blood vessels. Specifically, remarkable functional ecto-AMPase activity was detected in odontoblasts (Fig. 1A1, A2). Rampant ecto-AMPase activity was also detected in the subodontoblast region, where Rashaok nerve plexus and blood vessels were located (Fig. 1A1, A2). Slight positive staining for ecto-AMPase activity was also detected in dental pulp blood vessels (Fig. 1A1, A3). Furthermore, we demonstrated that some dental pulp nerve fibers were positive for ecto-AMPase activity staining (Fig. 1A4). In a negative control experiment, no positive ecto-AMPase staining was detected as AMP was omitted from the incubation solution (data not shown).

We further tested the ecto-AMPase activity in TG. As shown in Figure 1B, positive staining for ecto-AMPase activity was detected in sections from TG. Specifically, ecto-AMPase activity was present in the TG neurons (Fig. 1B1, B2) as well as in their axonal fibers in TG (Fig. 1B1, B3). The staining for ecto-AMPase activity in TG neurons was not homogeneous. Some neurons displayed strong positive staining, while others displayed weak positive or even negative staining (Fig. 1B1, B2). The percentage of cells that displayed strong ecto-AMPase activity in TG was about 23% (a total of

269 cells were counted in 4 different slices). In addition, we also detected positive staining for ecto-AMPase activity in trigeminal nerve fibers that project to the brainstem (Fig. 1B4). Detection of functional ecto-AMPase activity in dental pulp nerve fibers and in TG neurons suggests that ecto-nucleotidases responsible for AMP hydrolysis might be involved in the modulation of dental orofacial pain.

Expression of CD73 in TG Nociceptive Neurons

Previous work has shown that CD73 is expressed in spinal cord dorsal root ganglia (Vongtau et al. 2011). We then tested if CD73 is expressed in TG neurons. As shown in Figure 2A, positive immunostaining for CD73 was detectable in TG cells (Fig. 2A1). To confirm that CD73 is expressed in TG neurons, coimmunostaining with neuronal marker MAP2 was performed (Fig. 2A2). As expected, most of the CD73-positive cells in the TG were colocalized with MAP2 staining (Fig. 2A3). Similar to the staining pattern for ecto-AMPase activity, the expression of CD73 in TG neurons was not homogeneous but displayed a mosaic pattern. We noted that some CD73-positive cells expressed a strong staining signal, which accounted for 25% of the total TG cells. Interestingly, some of the strong CD73-positive cells colocalized with strong neuronal marker MAP2 staining, while some expressed weak MAP2 staining, and few were negative for MAP2 staining (Fig. 2A). In contrast, all of the CD73 weak positive staining cells were colocalized with MAP2 staining in the TG. These observations suggest the variation of CD73 expression in TG neurons. Besides expression in TG neurons, CD73 immunostaining signal was also detected in the nerve fibers. We observed positive CD73 staining in nerve fibers within the TG (Fig. 2A) as well as in the nerve trunks projecting to the brainstem (data not shown).

To demonstrate that CD73-positive staining neurons in TG are nociceptive neurons, we made costaining with nociceptive neuronal marker IB4. As shown in Figure 2B, almost all of the IB4-positive staining neurons overlapped with CD73-positive staining in the TG (Fig. 2). We also demonstrated that IB4 staining is colocalized with CD73 staining in trigeminal nerve trunks projecting to the brainstem (Fig. 2C). We noted that some CD73-positive staining axonal fibers were negative for IB4 staining (Fig. 2C), which is consistent with the observation that

Figure 1. Detection of ecto-AMPase activity in dental pulps and trigeminal nerves. **(A)** Histochemical staining for ecto-AMPase activity in human dental pulp. (A1) Positive staining was detected in dental pulp odontoblast layer, subodontoblast layer, and blood vessels. (A2) Positive staining for ecto-AMPase activity in odontoblasts and subodontoblast layer. (A3) Positive staining for ecto-AMPase activity in dental pulp blood vessels. (A4) Ecto-AMPase activity positive staining in dental pulp nerve fibers. BV, blood vessels; NF, dental pulp nerve fibers; OB, odontoblasts; Sub-OB, subodontoblast layer. Scale bars: 20, 10, 10, and 10 μ m, respectively. **(B)** Histochemical staining for ecto-AMPase activity in trigeminal ganglia (TG). (B1) Positive staining in presumable TG neurons (arrowheads) and their nerve fibers (open triangles) were detected in TG. (B2) Mosaic pattern of ecto-AMPase activity staining in TG neurons. Arrows: Strong positive staining neurons. Arrowheads: Weak positive staining neurons. Open triangles: Negative staining neurons. (B3) Detection of ecto-AMPase activity in the same TG neuron and its axonal nerve fiber. Arrow: Positive staining in a TG neuron. Open triangle: Positive staining in the initial segment of its axonal fiber. (B4) Detection of ecto-AMPase activity in trigeminal central axonal fibers. Open triangles: Positive staining for ecto-AMPase activity in trigeminal axonal fibers from nerve trunks projecting to the brainstem. Scale bars: 15, 10, 15, and 10 μ m, respectively.

only a portion of the nociceptive axonal fibers binds IB4 (Bae et al. 2015). The presence of CD73 in nociceptive axonal fibers projecting to the brainstem suggests that CD73 might be transported to the presynaptic terminals in the nociceptive lamina in the (TSNC).

Detection of Ecto-AMPase Activity in the Outermost Layer of the TSNC

Ecto-nucleotidases responsible for AMP hydrolysis have been detected in the nociceptive lamina in the dorsal horns of the spinal cord. It is not clear if ecto-AMPases are present in the corresponding nociceptive lamina of the TSNC in the brainstem (Fig. 3A). We first tested the functional ecto-AMPase activity in the brainstem. As illustrated in Figure 3B, C, ecto-AMPase activity was detected in the outermost layer of the TSNC, which

Figure 2. Expression of CD73 in trigeminal ganglia (TG). (A) Immunofluorescence staining for CD73 in the TG. (A1) Positive staining for CD73 was detected in TG cells. (A2) Coimmunostaining for neuronal marker MAP2 in the TG. The TG neurons were identified by positive staining of MAP2. (A3) Colocalization of CD73 and MAP2 staining in TG neurons (arrows or arrowheads) and their axonal nerve fibers (open triangles). Strong and weak CD73 positive staining were identified. Arrows: Strong CD73-positive staining neurons. Arrowheads: Weak CD73-positive staining neurons. Most of the weak CD73-positive staining cells were colocalized with MAP2 staining in the TG. For strong CD73-positive staining, some were colocalized with MAP2 staining (arrowheads), while others were negative for MAP2 staining (arrows). Open triangles: CD73-positive staining in axonal fibers. (B) Expression of CD73 in TG nociceptive neurons. (B1) Immunostaining for CD73 in the TG. (B2) Costaining for nociceptive neuronal marker IB4. (B3) Colocalization of the CD73 staining and IB4 staining in the TG. All of the CD73-positive staining neurons, including both the strong (arrows) and weak (arrow heads) positive staining neurons, were colocalized with IB4 staining in the TG. (C) CD73 was detectable in TG nociceptive axonal fibers projecting to the brainstem. (C1) Positive staining for CD73 was detected in trigeminal nerve axonal fibers. (C2) Nociceptive neuronal marker IB4 was also detected in the trigeminal nerve axonal fibers. (C3) Colocalization of CD73 and IB4 staining in trigeminal axonal fibers projecting to the brainstem. Open triangle: Colocalization of CD73 and IB4 staining in the axonal fibers. Arrowheads: CD73-positive staining and IB4-negative staining axonal fibers. Scale bars: 10 μ m for A, B, and C, respectively.

corresponds to the nociceptive lamina I/II in the dorsal horns of the spinal cord. To confirm the similar existence of ecto-AMPase activity in the spinal cord, the same protocol was applied to section of spinal cord, as shown in Figure 3D; positive staining in the outermost layer of the dorsal horn was also detected. Since AMP is usually generated from hydrolysis of ATP and ADP, we then tested the existence of ecto-ADPase and ecto-ATPase activity in the nociceptive lamina. As expected,

Figure 3. Detection of ecto-AMPase activity in the trigeminal subnucleus caudalis (TSNC) in the brainstem and the upper cervical spinal cord. (A) Schematic shows the location of the TSNC in the brainstem. (B) Positive staining for ecto-AMPase activity was detected in the outermost layer of the TSNC in the region corresponding to the rectangle (a) marked in A. (C) Positive staining for ecto-AMPase activity in the TSNC in the region corresponding to the rectangle (b) marked in A. (D) Ecto-AMPase activity staining in the upper cervical spinal cord. Similar positive staining for ecto-AMPase activity was also detected in the outermost layer of the upper cervical spinal cord dorsal horn. (E) Ecto-ADPase activity staining in the upper cervical spinal cord. Positive staining for ecto-ADPase activity was also detected in the outermost layer of the upper cervical spinal cord dorsal horn.

both ecto-ADPase activity (Fig. 3D) and ecto-ATPase activity (data not shown) were detected in the nociceptive lamina. Our results indicate that in the outermost layers of the TSNC or the dorsal horn of the spinal cord, there exist machineries for adenosine generation via nucleotide hydrolysis.

Detection of CD73 in the Nociceptive Lamina of the TSNC

The primary nociceptive sensory neurons send axonal fibers to form synapses with the secondary neurons in the brainstem or spinal cord. It has been well established that the nociceptive A δ and c fibers project to the outermost layers (lamina I/II) of the dorsal horn. To identify the presence of CD73 in the TSNC, we performed double immunostaining for CD73 and MAP2 in slices sectioned from the brainstem. As expected, positive staining for CD73 was detected in the outermost layers of the TSNC (Fig. 4A). To confirm the presence of CD73 in the brainstem is located in the nociceptive lamina of the TSNC, we performed costaining with nociceptive neuronal marker IB4. As expected, CD73 staining overlapped with the IB4 staining (Fig. 4B) in the outermost layer of the TSNC, corresponding to the nociceptive lamina (I/II) in the dorsal horn of the spinal cord. The characterized plaque or punctate staining pattern for CD73 and its colocalization with IB4 staining in the outermost layer of the TSNC suggest that CD73 is most likely localized in the presynaptic axonal terminals.

Figure 4. Detection of CD73 in the trigeminal subnucleus caudalis (TSBC) in the brainstem. **(A)** CD73 was detected in the outermost layer of the TSBC in the brainstem. (A1) Positive immunostaining for CD73 was detected in coronal section from the brainstem. (A2) Immunostaining for neuronal marker MAP2 in coronal section from the brainstem. (A3) Coimmunostaining for CD73 and MAP2 in the brainstem demonstrates the localization of CD73 in the brainstem, which corresponds to the outermost layer of the trigeminal sensory nuclear complex (TSNC). Scale bar: 40 μ m. **(B)** CD73 was detected in the nociceptive lamina of the TSBC in the brainstem. (B1) Positive immunostaining for CD73 in the coronal section from the brainstem. (B2) Staining for nociceptive neuronal marker IB4 in the coronal section from the brainstem. The positive IB4 staining region represents the nociceptive lamina in the brainstem. (B3) Overlapping of CD73 and IB4 staining in the brainstem demonstrating the nociceptive lamina localization of CD73 in the TSNC in the brainstem. Scale bar: 40 μ m.

Incubation with Specific Anti-CD73 Serum Reduces Extracellular Adenosine Generation in Trigeminal Nociceptive Lamina

The presence of CD73 in primary nociceptive neurons and the nociceptive lamina of the TSNC suggests that CD73 may participate in nociception modulation by controlling endogenous adenosine generation in orofacial pain. We further tested if CD73 is responsible for extracellular adenosine generation in the trigeminal nociceptive pathway. To demonstrate that CD73 is involved in extracellular adenosine generation, we tested if incubation with specific anti-CD73 serum could abolish the ecto-AMPase activity staining in the brainstem near the cervical cord level. As shown in Figure 5A, B, compared to the control, staining for ecto-AMPase activity in the outermost layers of the TSNC was reduced after incubation with anti-CD73 serum. In contrast, we noted that the staining in blood vessels was not reduced but even slightly enhanced after incubation with anti-CD73 serum (Fig. 5B). Figure 5C shows the statistical results for the effect of anti-CD73 serum incubation on ecto-AMPase activity in the nociceptive lamina and in blood vessels, respectively. After incubation with the specific

Figure 5. CD73 participates in extracellular adenosine generation in the nociceptive lamina. **(A)** Incubation with specific anti-CD73 serum reduces ecto-AMPase activity staining in the nociceptive lamina. (A1) Enzymatic histochemical detection of ecto-AMPase activity in the brainstem/upper cervical spinal cord. Positive staining for ecto-AMPase activity was detected in the outermost layer of the TSNC/dorsal horn in the control. (A2) Effects of preincubation with specific anti-CD73 serum on ecto-AMPase activity staining. The ecto-AMPase activity staining in the nociceptive lamina was reduced after incubation with anti-CD73 serum (arrows). In contrast, ecto-AMPase activity staining in the blood vessels was less affected or even was enhanced in the same slices (arrowheads). **(B)** Statistical analysis for the changes of ecto-AMPase activity staining in slices after incubation with either control serum (control) or anti-CD73 serum (blocking). The average staining intensity of ecto-AMPase activity either in the nociceptive lamina or in blood vessels was quantified and adjusted with the background insensitivity. Unpaired Student's *t* test. $^{**}P < 0.001$, $n = 10$ represents slice number used in each group.

anti-CD73 serum, ecto-AMPase activity in the nociceptive lamina was reduced by 67% ($P < 0.001$), while ecto-AMPase staining in blood vessels was increased by 3.6%, although there is no statistically significant difference ($P = 0.073$).

Discussion

Purinergic signaling is involved in pain signal transduction, transmission, modulation, and sensitization in the nociceptive sensory nervous system (Hamilton 2002; Wirkner et al. 2007; Fabbretti 2013). It has been well established that endogenous adenosine induces antinociception in various pain models (Sawynok 2015). Adenosine induces analgesia via activation of A1 receptors expressed in the peripheral sensory nerve fibers (Lima et al. 2010). It has been demonstrated that blocking A1 receptors abolishes adenosine-induced antinociception

in various inflammatory pain models (Maes et al. 2012). In addition, adenosine also mediates the analgesic mechanism of acupuncture via peripheral A1 receptors (Goldman et al. 2010). Besides the peripheral mechanism, adenosine also mediates antinociception via A1 receptors in the spinal cord (Lima et al. 2010). Intraspinal injection of adenosine or A1 receptor agonists induced antinociception in both inflammatory and neuropathic pain animal models. Activation of A1 receptor induces ion channel (Ca^{2+} and K^{+}) expression, receptor uncoupling, and downregulation by triggering multiple intracellular signal cascades, including cAMP/PKA, PLC/IP3/DAG, and nitric oxide/cGMP/PKG pathways (Lima et al. 2010; Chen et al. 2014). The underlying antinociceptive mechanism includes potassium channel activation-induced cell membrane hyperpolarization (Patel et al. 2001) that decreases presynaptic vesicle release and reduces postsynaptic excitability. Indeed, it has been observed that A1 receptor activation reduces the excitatory neurotransmitter release in synaptosomes isolated from the dorsal horns of the spinal cord (Santicoli et al. 1992; Li and Eisenach 2005). Endogenous adenosine also mediates antinociception by activating A2A, A2B, or A3 receptors expressed in nociceptive neurons, astrocytes, or immune cells (Feoktistov and Biaggioni 2011; Borea et al. 2015). Considering the extensive involvements of glia cells (microglia, astrocytes) in central sensitization and chronic pain (Ji et al. 2013), activation of adenosine receptors on microglia and astrocytes are potentially involved in the antinociceptive mechanism of adenosine in the nervous system (Paterniti et al. 2011). In addition, adenosine may also mediate the antinociception by enhancing GABA inhibition and blocking neuroinflammation via activation of A3 receptors (Ford et al. 2015).

Adenosine-mediated antinociception depends on the extracellular adenosine level (Sawynok and Liu 2003). Even though adenosine transport (Maes et al. 2012) and metabolism (Schmidt et al. 2009) have been shown to participate in the pain modulation, ecto-nucleotidase-mediated AMP hydrolysis is a major player to control the extracellular adenosine levels (Street and Zylka 2011). In this study, we detected ecto-AMPase activity in the dental pulp odontoblast layer, subodontoblast nerve plexus, and dental pulp nerve fibers, which suggests that adenosine can be generated from AMP in dental pulp. Inflammation is usually accompanied by a large amount of ATP release and will be quickly hydrolyzed to AMP via NTPDases (Robson et al. 2006). Interestingly, our previous works have demonstrated the presence of ecto-ATPase activity and the expression of ecto-nucleotidases (NTPDase1, 2, 3) in dental pulp as well as TG (Liu et al. 2012; Liu et al. 2015). Together with the present observations, our results are consistent with the notion that machineries coexist in dental pulp to generate extracellular adenosine. It would be interesting to further investigate the potential role of adenosine signaling in the generation and mitigation of dentin hypersensitivity and dental pain.

The enzymes responsible for extracellular nucleotide hydrolysis may affect the pain signal transduction and modulation via purinergic signaling in the nociceptive pathways. Indeed, knockout of ecto-nucleotidases responsible for AMP hydrolysis enhances the nociception in inflammatory and

neuropathic pain models. Accordingly, intraspinal application of soluble isoform nucleotidases responsible for AMP hydrolysis induced significant long-term analgesia (Zylka 2011). Consistent with the detection of ecto-AMPase activity, we further confirmed the expression of CD73 on TG nociceptive neurons and in the nociceptive lamina of the TSNC in the brainstem. These observations support the notion that adenosine signaling is also present in trigeminal nociceptive nerves and may participate in the pain modulation in dental orofacial pain. Interestingly, it had been observed that extracellular adenosine and A1 receptor activation reduced presynaptic glutamate release and inhibited the excitatory postsynaptic activity in the brainstem nociceptive neurons (Kumar et al. 2012; Choi et al. 2015). Our anti-CD73 serum incubation experiment confirmed that CD73 plays a major role for extracellular adenosine generation (account for 67%) in the nociceptive lamina of the TSNC, but it also indicates the existence of alternative ecto-AMPases, which is consistent with the previous observation that prostatic acid phosphatase (PAP) also contributes to the adenosine generation in the dorsal horn of the spinal cord (Street et al. 2011). However, the exact profiles of ecto-nucleotidases responsible for adenosine generation in the nociceptive lamina in the brainstem and their role in the modulation of orofacial pain need to be further investigated.

Neuropathic pain is characterized by neuropathy changes induced by multiple assaults, including peripheral nerve deafferentation, local constriction, chronic inflammation, virus infection, metabolic disorders, and oxidant stress (Gilron et al. 2015). Our observations that ecto-AMPase activity and CD73 expression in the trigeminal nerve fibers projecting to the brainstem suggest that CD73 is most likely synthesized in TG neurons and is then transported to the presynaptic terminals at the brainstem. The specific localization of ecto-AMPase activity and the characterized punctate- and plaque-staining pattern of CD73 in the nociceptive lamina further support the presynaptic localization of CD73 in the brainstem. Disruption of CD73 axonal transportation to the presynaptic terminals may contribute to the development of neuropathic pain in trigeminal nerves. Recently, it has been observed that disruption of fast axonal transport induces behavioral changes consistent with neuropathic pain (Dilley et al. 2013). Since the presence of ecto-AMPases, including CD73, is responsible for tonic activation of A1 receptors in the nociceptive lamina (Street et al. 2011), we propose that various environmental assaults to the peripheral nerves may induce neuropathic pain by affecting the axonal transport of ecto-nucleotidases. Reducing neuronal ecto-nucleotidase, including NTPDas3, PAP, and CD73, in the presynaptic terminals will reduce pain mitigation and induce pain sensitization by increasing the local concentration of ATP and decreasing the generation of adenosine. Therefore, any physical, chemical, biological, and genetic factors that affect the localization of neuronal ecto-nucleotidases to peripheral and central terminals will affect the pain signal transduction, transmission, modulation, and sensitization in the nociceptive nervous system. The effects of trigeminal nerve fiber injury or constriction on presynaptic terminal localization of ecto-nucleotidases and its relation to the development of neuropathic pain await further studies.

Purinergic signaling participates in pain processing by affecting pain transduction, transmission, and modulation in the nociceptive sensory nerves. Ecto-nucleotidases play a strategic role in controlling the duration, magnitude, and chemical nature of the purinergic signals by hydrolyzing extracellular nucleotides. Specifically, ecto-nucleotidases in the nociceptive pathway drive a shift from ATP-driven pain sensation to an adenosine-driven antinociception. By controlling the generation of adenosine, the expression and localization of CD73 in trigeminal nociceptive neurons and presynaptic terminals are expected to play an important role in mitigating orofacial pain. Thus, potential drugs that alter this catabolic machinery will provide an exciting opportunity for improved therapeutic management of dental orofacial pain.

Author Contributions

X. Liu, contributed to conception and design, data acquisition, analysis, or interpretation, drafted and critically revised the manuscript; L. Ma, S. Zhang, contributed to data acquisition and analysis, drafted and critically revised the manuscript; Y. Ren, R.T. Dirksen, contributed to data interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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