

Chapter 13

TNAP and Pain Control

Sarah E. Street and Nathaniel A. Sowa

Abstract Chronic pain is one of the most debilitating and expensive diseases, yet current therapies are often insufficient in bringing about long-term relief. Further, many treatments for chronic pain also carry significant side effects. The molecule adenosine has long been identified as a potent inhibitor of nociceptive circuits in the spinal cord; however, the widespread expression of adenosine receptors in many organ systems has limited its use as an analgesic. Recently several 5' ectonucleotidases, including tissue non-specific alkaline phosphatase (TNAP), have been characterized for their ability to generate endogenous adenosine in nociceptive circuitry of the dorsal spinal cord. These ectonucleotidases have the ability to hydrolyze the endogenous pronociceptive nucleotides like adenosine triphosphate (ATP) into the antinociceptive nucleoside adenosine. This chapter discusses the role of TNAP and other ectonucleotidases in nociceptive circuits, and their potential as future targets of new therapeutics to treat chronic pain.

Keywords Tissue non-specific alkaline phosphatase (TNAP) · Nociception · Dorsal root ganglia · Adenosine · Ectonucleotidase

13.1 The Burden of Chronic Pain

Pain is one of the most common medical complaints in the United States. An estimated 100 million American adults suffer from chronic pain (Relieving Pain in America: A blueprint for transforming prevention, care, education and research

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2011). In one study, over one-fourth of all adults in the United States reported that they suffered from pain that persisted for 24 h, and 42 % of those individuals reported that their pain had lasted greater than one year (Health, United States 2006 With Chartbook of Trends in the Health of Americans 2006). Chronic pain can be extremely debilitating, affecting physical and emotional well-being, and patients who suffer from moderate to severe chronic pain report difficulty performing everyday activities, including exercising, sleeping, doing chores, participating in social activities and carrying out familial responsibilities (Chronic Pain in America: Roadblocks to Relief 1999). The total societal cost of chronic pain has been estimated to lie close to 635 billion dollars, which includes money for treatment and lost productivity (Relieving Pain in America: A blueprint for transforming prevention, care, education and research 2011).

Treatments for pain are not only expensive, but they often fall short of alleviating pain without significant side effects. Pharmaceutical treatments are the most common, and include non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase inhibitors, opioids, antidepressants, anticonvulsants and ion-channel blockers. While they are some of the most commonly prescribed drugs on the market, their concomitant side effects such as nausea, drowsiness, dependency, and tolerance make their long-term use problematic for most patients. Other modes of pain relief have subsequently gained popularity, such as injections of local anesthetics, nerve blocks, electrical stimulation, psychological therapy, surgery and even acupuncture. Even despite these treatments, many patients still suffer, necessitating further advances in pain therapy.

Over the past several years, enzymes collectively known as ectonucleotidases have emerged as potential new targets for pain treatment. Ectonucleotidases are extracellular enzymes that break down nucleotides, such as adenosine triphosphate (ATP) into nucleosides like adenosine (Hilaire et al. 2011; Zimmermann 2006). Tissue non-specific alkaline phosphatase (TNAP) is the most recent of three ectonucleotidases that have been characterized in nociceptive (pain-sensing) circuits of the dorsal horn in the spinal cord of mice (Street et al. 2013). TNAP recently joined prostatic acid phosphatase (PAP), and ecto-5'-nucleotidase (NT5E) as members of the family of ectonucleotidases whose function is necessary for normal nociceptive signaling in spinal cord circuits (Zylka 2011). This chapter will discuss how recent findings suggest TNAP's ectonucleotidase activity, along with those of PAP and NT5E, is essential in maintaining proper purinergic tone in nociceptive circuits. Further, it will highlight the potential ability to reduce pain that each of these ectonucleotidases harbor.

13.2 Nociceptive Circuitry in the DRG and Spinal Cord

Highly specialized neurons in the periphery, known as primary sensory neurons, provide information about the environment, including the presence of noxious stimuli, to the central nervous system through axonal fibers. Neurons that are specialized to detect noxious stimuli are called nociceptors (Sherrington 1906).

Some nociceptors can detect several forms of noxious stimuli, while others are specialized for one type of input (Julius and Basbaum 2001; Meyer et al. 2006). Fibers arising from these neurons have been shown, using electrophysiological techniques, to be excited by noxious heat and cold, intense pressure, and chemical irritants, but not by innocuous warming or light touch (Burgess and Perl 1967). These neurons house their pseudo-unipolar cell bodies in the trigeminal and dorsal root ganglia (DRG). They then send their central processes into the spinal cord or brainstem where they synapse with second-order sensory neurons. These neurons then either synapse on other neurons in the spinal cord and brainstem, or directly relay information to the thalamus (Meyer et al. 2006).

There are two broad classes of primary nociceptive neurons that encode nociceptive information. First, thinly myelinated A δ fibers arise from medium diameter cell bodies in the DRG or trigeminal ganglia. These sensory neurons detect noxious heat and mechanical stimuli, and play a role in the sensitization process following burn or chemical injury (Campbell et al. 1979; Dubner and Hu 1977; Meyer et al. 2006; Ringkamp et al. 2001; Treede et al. 1998). Second, small-diameter, unmyelinated C-fibers respond to the same noxious stimuli, but propagate action potentials more slowly, display slowly adapting responses to stimuli, and are heavily involved in the sensitization process following tissue injury (Meyer et al. 2006; Peng et al. 2003).

These small nociceptive DRG neurons are further subdivided into classification based on cellular markers (Woolf and Ma 2007). Peptidergic neurons contain neuropeptides such as calcitonin gene-related protein (CGRP), substance P, and somatostatin, and also express TrkA, the tyrosine kinase receptors that bind nerve growth factor (Fitzgerald 2005; Julius and Basbaum 2001). These neurons predominantly project to lamina I and the outer segment of lamina II in the dorsal horn (Hunt and Mantyh 2001; Zylka et al. 2005). Nonpeptidergic neurons express proteins such as PAP, the G-protein coupled receptors MrgprD, and the ATP-gated ion channel P2X₃, bind the plant lectin IB4 (Meyer et al. 2006; Silverman and Kruger 1988a, b; Snider and McMahon 1998; Vulchanova et al. 1998; Zylka et al. 2005, 2008), and project to the inner layer of lamina II (Perry and Lawson 1998; Zylka et al. 2005). These gene expression patterns also loosely translate to the segregation of nociceptive sensory input. For instance, the heat- and capsaicin-sensitive channel TRPV1 is mostly expressed in peptidergic neurons (Cavanaugh et al. 2009; McCoy et al. 2012), whereas nonpeptidergic neurons that express MrgprD seem to encode information regarding mechanical stimuli (Cavanaugh et al. 2009). Thus, these categories also have some functional implication.

13.3 ATP and Adenosine's Role in Nociception

ATP and adenosine both play important roles in nociception. ATP is released by nearly all cells, including neurons and glia in response to both physiological and pathological stimuli (Arcuino et al. 2002; Gourine et al. 2010; Matsuka et al. 2008;

Nakamura and Strittmatter 1996). ATP generally exerts its effects through purinergic P2 receptors, of which there are two families—P2X and P2Y. P2X receptors are ligand-gated, cation-selective channels that lead to excitation and pro-nociceptive effects, while P2Y receptors are G-protein coupled receptors (GPCRs) that have been reported to have both pro- and anti-nociceptive effects (Burnstock 2009; Gerevich et al. 2005, 2007; Moriyama et al. 2003; Okada et al. 2002; Sawynok et al. 2006; Tominaga et al. 2001). Of the seven cloned mammalian P2X receptors, P2X₃ is the most abundant in DRG neurons. P2X₃ receptors colocalize with markers of nonpeptidergic sensory neurons such as MrgprD and PAP (Zylka et al. 2005, 2008). Further, administration of ATP or other P2X₃ receptors agonists result in excitation of C-fibers and pain-avoiding behaviors such as thermal hyperalgesia and mechanical allodynia (Dowd et al. 1998; Hamilton et al. 2001, 1999; Hilliges et al. 2002; Sawynok and Reid 1997; Tsuda et al. 2000). This is not surprising since inflammation and nerve injury lead to upregulation of P2X₃ expression in DRG and spinal cord neurons, and sensitize the receptor to ATP through phosphorylation (Chen et al. 2001; Dai et al. 2004; Novakovic et al. 1999; Paukert et al. 2001; Zhou et al. 2001; Xu and Huang 2002). In addition to P2X₃ receptors, P2X_{2/3} and P2X₄ receptors also play a pro-nociceptive role in spinal sensory circuits (Nakagawa et al. 2007; Tsuda et al. 2003). Thus, ATP, in general, has a pro-nociceptive effect through its activation of P2X receptors.

On the other hand, adenosine, a product of ATP hydrolysis, exerts a potent antinociceptive effect. Adenosine activates four subtypes of P1 receptors—A₁, A_{2A}, A_{2B}, and A₃ (Abbracchio et al. 2009; Burnstock 2009). Each of these P1 receptors is a GPCR that couple to different types of G proteins. The A₁ adenosine receptor (A₁R) is expressed by small and medium diameter DRG and trigeminal ganglion neurons, as well as postsynaptic sites in the dorsal horn of the spinal cord and brainstem (Carruthers et al. 2001; Schulte et al. 2003). Activation of A₁R results in reduced Ca²⁺ entry, decreased substance P and CGRP release from DRG neurons, as well as inhibition of neurotransmission through both pre- and post-synaptic mechanisms in the spinal cord (Carruthers et al. 2001; Haas and Selbach 2000; Li and Perl 1994; Patel et al. 2001; Santicoli et al. 1993; Sjolund et al. 1997).

Activation of A₁R by adenosine or other agonists administered both peripherally and centrally causes antinociceptive effects. For instance, local administration of A₁R agonists into the rat hind paw leads to a decrease in nociceptive behavior in several different models of inflammatory and neuropathic pain (Aley et al. 1995; Aumeerally et al. 2004; Karlsten et al. 1992; Liu et al. 2000; Taiwo and Levine 1990). Also, spinal application of adenosine, A₁R agonists, or other agents that increase the endogenous adenosine levels have antinociceptive effects in animal models of chronic pain (Dickenson et al. 2000; Sawynok and Liu 2003; Zylka 2011). Conversely, mice lacking A₁R (A₁R^{-/-}) show enhanced nociception including thermal sensitivity and thermal hyperalgesia, but have normal responses to mechanical stimuli (Johansson et al. 2001; Wu et al. 2005). Finally, the antinociceptive effect of acupuncture is dependent upon adenosine generation and

activation of A₁R (Goldman et al. 2010). Taken together, these data suggest great potential for targeting of A₁R for treatment of chronic pain. However, studies using high doses of A₁R-selective agonists have shown side effects including motor paralysis and autonomic dysfunction (reviewed in Zylka 2011). Thus, in order to harness the potential antinociceptive action of A₁R, a more indirect mode of activation must be determined.

13.4 Ectonucleotidases and Extracellular Adenosine Generation

One source of extracellular adenosine results from the breakdown of nucleotides such as ATP, ADP and AMP. This extracellular production of adenosine relies on the activity of several different enzymes that degrade amine-containing nucleotides in a step-wise manner. While some enzymes hydrolyze ATP, ADP and AMP, others are specific for a particular adenine-containing nucleotide. These enzymes, known generally as ectonucleotidases, play important roles in the regulation of both P1 receptors (through the production of adenosine) and P2 receptors (through the degradation of ATP) activity (Sawynok 2007; Zimmermann 2006). Since their activity can decrease activation of the pronociceptive P2X receptors, and increase the activation of antinociceptive A₁R receptors, ectonucleotidases sit at a pivotal position in the regulation of nociceptive circuits.

Prior to the discovery of TNAP as an important ectonucleotidase in nociceptive signaling, two other ectonucleotidases were previously identified and studied in the context of pain control in mice. First, a long-standing question in neuroscience was answered when the molecular identity of the fluoride-resistant acid phosphatase, used to identify a sub-population of small-diameter DRG neurons, was shown to be the ectonucleotidase PAP (Zylka et al. 2008). PAP is an acid phosphatase that hydrolyzes AMP into adenosine. In nociceptive circuits, PAP is predominantly expressed in nonpeptidergic DRG neurons, and in their central terminals in lamina II. What was perhaps the most surprising finding of this study however, was spinal injection of PAP led to potent antinociceptive effects in animal models of neuropathic and chronic inflammatory pain (Sowa et al. 2009; Zylka et al. 2008). Further, these antinociceptive effects lasted three-times longer than morphine without any of the apparent side effects. Interestingly, mice lacking A₁R did not show any antinociceptive effects after spinal injection of PAP, suggesting that this antinociceptive activity of PAP was dependent upon the generation of adenosine. Additionally, mice lacking PAP (*Pap*^{-/-}) showed hyperalgesia and mechanical allodynia (sensitization of mechanical sensory neurons) in models of chronic inflammatory pain (Zylka et al. 2008). However, AMP hydrolytic activity was only partly reduced in *Pap*^{-/-} mice, suggesting that there was at least one other ectonucleotidase responsible for breaking down AMP.

Shortly after PAP was demonstrated to be an important player in nociceptive circuitry, NT5E, an ectonucleotidase that also hydrolyzes AMP into adenosine, was shown to be expressed in DRG neurons (Sowa et al. 2010b). While PAP colocalizes predominantly with nonpeptidergic DRG neurons, NT5E is more broadly expressed across all classes of nociceptive neurons. Similar to PAP, mice lacking NT5E (*Nt5e*^{-/-}) showed enhanced nociceptive sensitivity in several behavioral models of pain, and spinal injection of NT5E protein caused anti-nociceptive behavior (Sowa et al. 2010b, c). However, when NT5E was injected into mice lacking A₁R, the anti-nociceptive effect was abolished (Sowa et al. 2010c). Further, when AMP and an adenosine transporter inhibitor were injected into the spinal cords of mice, *Nt5e*^{-/-} mice demonstrated a 50 % reduction in antinociceptive behavior compared to the control (Sowa et al. 2010b). These data suggested that, like PAP, NT5E also was not solely responsible for adenosine generation in nociceptive circuits. Indeed, AMP hydrolysis was decreased, but not abolished in DRG and spinal cord sections from *Nt5e*^{-/-} mice, suggesting that both PAP and NT5E hydrolyze AMP and generate adenosine in the DRG and dorsal horn (Sowa et al. 2010b).

Double knockout (dKO) mice that lack both PAP and NT5E were subsequently bred to determine the extent to which these ectonucleotidases contribute to adenosine generation in the dorsal horn. While AMP hydrolysis was mostly reduced in the dKO mice at both physiological and acidic pH, behavioral studies showed that some AMP hydrolytic activity remained even after PAP and NT5E activity was eliminated (Street et al. 2011). Both *Pap*^{-/-} and *Nt5e*^{-/-} single knockout mice showed an approximately 50 % decrease in antinociceptive behavior when injected with AMP and an inhibitor of the adenosine transporter. However, when the same injection was made in the dKO mouse, a small but significant antinociceptive effect remained, suggesting that there was at least one additional remaining ectonucleotidase in spinal nociceptive circuits.

In an attempt to determine the remaining source of adenosine production in the dKO mice, fast-scan cyclic voltametry (FSCV) was used. FSCV enables the direct measurement of subsecond changes in extracellular adenosine concentration and can be done in mouse spinal cord slices (Swamy and Venton 2007). In this technique a carbon electrode measures the current generated when adenosine changes oxidation states when presented with voltage ramps from -0.5 to 1.5 mV. By measuring the current level generated at 1.0 mV, the voltage at which adenosine is oxidized, the concentration of adenosine can be deduced. Surprisingly, experiments using FSCV showed that even in spinal cord slices from dKO mice, about 30 % of maximum adenosine generation remained (Street et al. 2011). Further, spontaneous transient increases in adenosine concentration were decreased, but not eliminated in the dorsal horn of spinal cord slices from dKO mice (Street et al. 2011). These data strongly suggested that while PAP and NT5E are the predominate contributors to adenosine generation in the dorsal horn, there is at least one remaining ectonucleotidase that is important for the antinociceptive effects of adenosine generation.

13.5 The Role of TNAP in Nociceptive Circuits

FSCV experiments done in dKO animals at neutral pH suggested that AMP hydrolytic activity remained even after genetic deletion of both PAP and NT5E. However, when these experiments were carried out at pH 5.6, virtually no adenosine was detected, suggesting that the remaining ectonucleotidase was not active in acidic environments (Street et al. 2011). These findings led to the hypothesis that perhaps TNAP could be the remaining ectonucleotidase in the dorsal horn of the spinal cord. TNAP can hydrolyze the nucleotides ATP, ADP and AMP to generate adenosine at both physiological and alkaline pH (Ciancaglini et al. 2010; Scheibe et al. 2000; Zimmermann 2006). Further, TNAP has been shown to be widely expressed in the central nervous system, including the developing spinal cord (Fonta et al. 2004; Langer et al. 2008; MacGregor et al. 1995; Narisawa et al. 1994). However, TNAP had not previously been shown to be expressed in, or have a role in adult nociceptive circuits. Thus, the elucidation of TNAP's potential role in nociception was essential to further address the role of ectonucleotidases in the DRG and dorsal horn.

13.6 TNAP Gene Expression in DRG and Spinal Cord

First, to determine whether the TNAP gene was expressed in adult mice, *in situ* hybridization was performed on adult mouse DRG and spinal cord tissue. TNAP mRNA was detected in nearly all DRG neurons and in neurons throughout the dorsal horn of the spinal cord (Street et al. 2013). This expression pattern slightly differed from PAP and NT5E, since these ectonucleotidases were expressed in subsets of nociceptive and non-nociceptive DRG neurons (Sowa et al. 2010b; Zylka et al. 2008). Further, whereas PAP and NT5E protein expression in the dorsal horn comes primarily from DRG nerve terminals, the TNAP *in situ* hybridization results suggested that TNAP was expressed in neuronal cell bodies in lamina I and II. These results show that the broad expression of TNAP reported in the developing spinal cord continues into adulthood (MacGregor et al. 1995; Narisawa et al. 1994).

Since TNAP is expressed broadly in many subtypes of DRG neurons, it is likely co-expressed in small- and medium-diameter nociceptive sensory neurons, suggesting that TNAP, NT5E and PAP serve redundant roles in their modulation of nociception. Further, TNAP's localization pattern also suggests that it might serve as an ectonucleotidase in other sensory circuits beyond those activated by noxious stimuli. The level of TNAP expression did not increase in *Pap*^{-/-} and *Nt5e*^{-/-} single knockout mice or dKO mice, demonstrating that TNAP expression is not upregulated in response to decreased expression of other ectonucleotidases. Thus, baseline expression of TNAP is adequate to generate enough adenosine to carry out the remaining antinociceptive effects observed in previous experiments (Street et al. 2011, 2013).

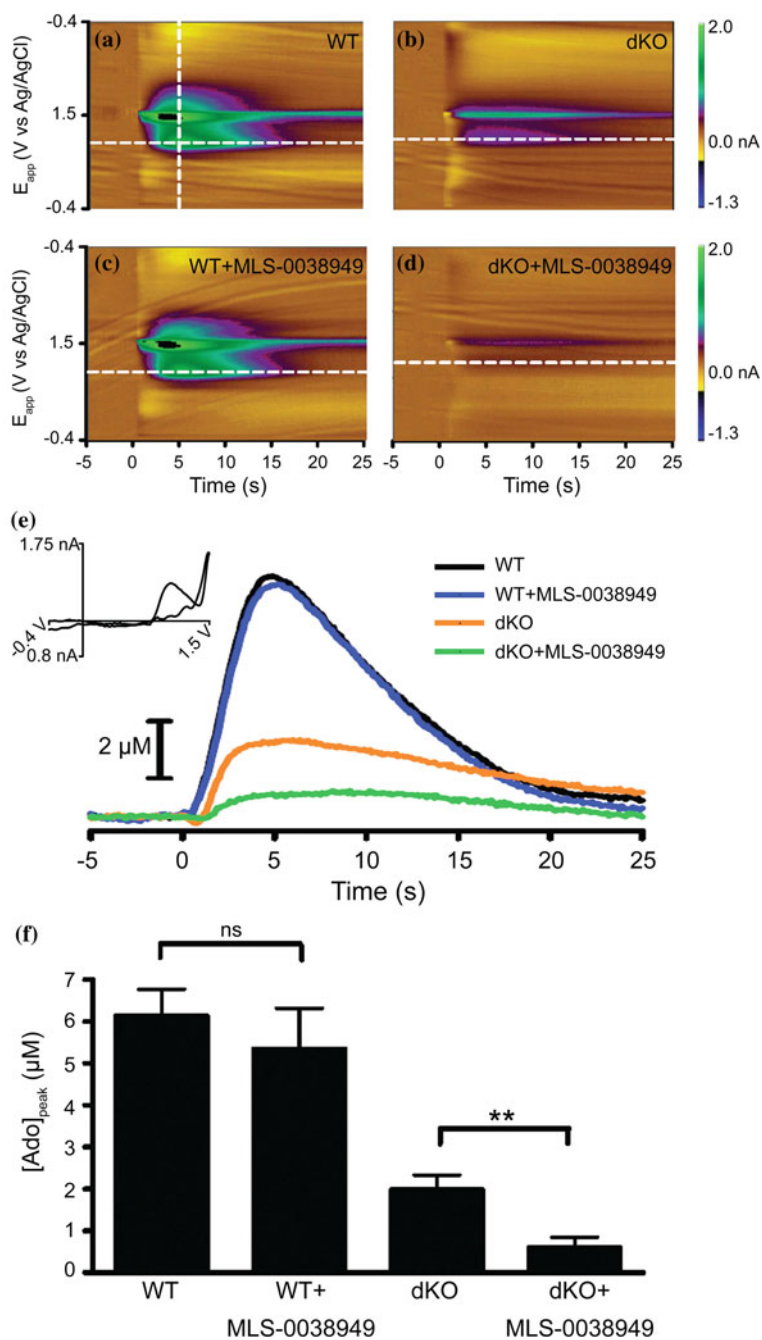
13.7 TNAP and Adenosine Generation in Nociceptive Circuits

TNAP, despite being more well known in the nervous system for its hydrolysis of pyridoxal-5' phosphate and its role in GABA metabolism, also breaks down extracellular nucleotides into adenosine. This ectonucleotidase activity has been reported in the hippocampus, where it plays a role in axon guidance, and also where it serves as the source of adenosine in the absence of NT5E (Diez-Zaera et al. 2011; Zhang et al. 2012). Unfortunately due to TNAP's role in GABA metabolism, *Tnap*^{-/-} mice develop seizures and die approximately 2 weeks after birth due to their inability to generate GABA, making them difficult to study (Narisawa et al. 1997; Waymire et al. 1995). However an inhibitor of TNAP, MLS-0038949, was used to study the role of TNAP in adenosine generation (Dahl et al. 2009; Sergienko et al. 2009). Indeed, MLS-0038949 blocked the activation of A_{2B}R when AMP was applied to HEK cells that expressed TNAP and A_{2B}R, showing that it completely inhibits the ectonucleotidase activity of TNAP (Street et al. 2013).

Since TNAP activity was adequately abolished with MLS-0038949, this compound was used to inhibit TNAP activity in AMP histochemistry studies in both DRG and spinal cord sections. Staining of AMP hydrolytic activity was reduced in both DRG and spinal cord in sections from dKO mice, but a substantial amount of activity remained, especially at alkaline pH (Street et al. 2013). However, at both physiological and alkaline pH, AMP hydrolytic activity in slices from dKO mice was completely abolished when MLS-0038949 was present, suggesting that TNAP was the sole remaining ectonucleotidase present in spinal nociceptive circuits.

In order to ensure that MLS-0038949 was specific to TNAP and had not inhibited another unidentified ectonucleotidase, AMP histochemistry was carried out on tissue from an early postnatal *Tnap*^{-/-} mouse. At pH 8.5, both DRG and spinal cord sections showed reduced staining in tissue from *Tnap*^{-/-} mice. To show that the residual histochemical staining was due to PAP and NT5E, we then treated WT and *Tnap*^{-/-} slices with tartrate and α , β methylene ADP, inhibitors of PAP and NT5E respectively. Again, when activity of all three ectonucleotidases was absent, AMP hydrolytic activity was eliminated (Street et al. 2013).

FSCV was then used to quantify the precise levels of adenosine generated in spinal cord slices that lack all three ectonucleotidases (Street et al. 2011). Similar to previous findings, when 100 mM AMP was applied to lamina I and II, a rapid transient increase in adenosine was detected by the electrode (Fig. 13.1a), and this increase in adenosine concentration was decreased in slices from dKO mice (Fig. 13.1b). However, in the presence of MLS-0028949 adenosine generation was dramatically reduced in dKO slices, but not in WT slices, suggesting that TNAP is most likely the last remaining ectonucleotidase expressed in the dorsal horn (Fig. 13.1c, d). When the concentration of the remaining adenosine was quantified, less than 1 μ M of adenosine was produced from a 100 mM bolus of AMP (Fig. 13.1e, f). It is also possible that some of this remaining adenosine was the result of spontaneous AMP degradation in the application solution. Since the EC₅₀ for adenosine binding to A₁R is approximately 1 μ M,



◀ **Fig. 13.1** Inhibition of TNAP, NT5E and PAP abolishes adenosine generation in the dorsal spinal cord. Fast scan cyclic voltametry (FSCV) was used to measure adenosine production at subsecond resolution. (a–d) FSCV color plots showing the level of current (color scale) that was generated over time (x-axis) and voltage (y-axis). 100 μ M AMP was pressure ejected for 1 s onto lamina II of (a, c) WT or (b, d) dKO mice in the absence or presence of MLS-0038949 (50 μ M). (e) Adenosine concentration, calculated from 1.0 V current (dashed horizontal lines in a–d). Inset, cyclic voltammogram confirms adenosine was produced (plotted from *dashed vertical line* in a). (f) Peak adenosine concentration after pressure ejecting AMP onto lamina II (n = 5 slices for each condition). **P < 0.005. Figure reprinted from Street et al. (2013) with permission

it is unlikely that the concentration of adenosine generated in slices lacking PAP, NT5E, and TNAP is enough to substantially activate A_1 Rs (Rittiner et al. 2012). Thus, TNAP is most likely the final ectonucleotidase responsible for the hydrolysis of AMP in the dorsal horn.

13.8 Functional Effects of TNAP Inhibition in Spinal Cord Slices

Since nociceptive DRG neurons synapse onto their target neurons primarily in lamina I and lamina II in the dorsal horn, synaptic neurotransmission between primary and secondary nociceptive neurons can be assessed by measuring the field excitatory post-synaptic potential (fEPSP) generated in lumbar dorsal horn slices after electrical stimulation of a lumbar nerve root (Ruscheweyh and Sandkuhler 2000; Street et al. 2011). AMP and adenosine both inhibit neurotransmission in the dorsal horn through activation of A_1 R, although AMP's action has previously been assumed to be indirect through the activity of ectonucleotidase (Dunwiddie et al. 1997; Lao et al. 2001; Li and Perl 1994, 1995; Salter and Henry 1985). Given these previous findings, electrophysiology was then used to determine the functional effects of inhibition of PAP, NT5E, and TNAP (Street et al. 2013).

As previously shown, both AMP and adenosine inhibit the $A\delta$ fEPSP strength in lamina II (Fig. 13.2a). This inhibition is dependent on A_1 R since there was no effect of AMP or adenosine in slices from $A_1R^{-/-}$ mice (Fig. 13.2b). AMP and adenosine was then applied to slices from $Pap^{-/-}$, $Nt5e^{-/-}$ and dKO mice. Surprisingly, there was no difference in the inhibitory effect of AMP on fEPSP strength in slices from any of the knockout mice, suggesting that either AMP could activate A_1 R without first being hydrolyzed to adenosine or that deletion of all three ectonucleotidases was required to sufficiently reduce adenosine generation (Fig. 13.2c). However, when slices from the different strains of mice were incubated in MLS-0038949, the inhibitory effect of AMP was decreased in slices from $Nt5e^{-/-}$ mice, and completely abolished in slices from dKO mice (Fig. 13.2d, e). Since inhibition of all three ectonucleotidases completely reduced AMP's effect on synaptic transmission, these data confirmed that AMP must first be hydrolyzed to adenosine in order to activate A_1 R and cause synaptic inhibition.

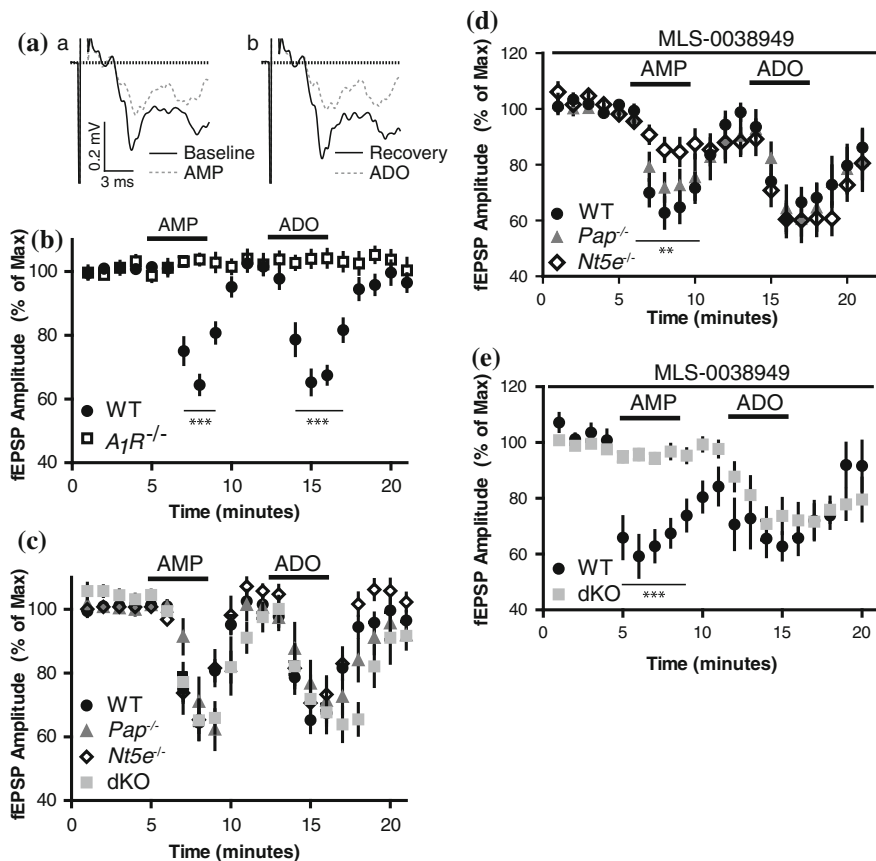


Fig. 13.2 TNAP, NT5E, and PAP are triply redundant in nociceptive circuits of the dorsal spinal cord. **a** Representative A δ fEPSPs in lamina II of WT spinal cord slices before (*solid line*) and after (*dashed line*) addition of **a** 250 μ M AMP or **b** 250 μ M adenosine (ADO) to perfusate. **b** Normalized fEPSP amplitude in WT and $A_1R^{-/-}$ spinal cord slices ($n = 25$ and 11 ; respectively). *** $P < 0.0005$. **c** Normalized fEPSP amplitude in WT, $Pap^{-/-}$, $Nt5e^{-/-}$, and dKO spinal cord slices. ($n = 25$, 10 , 20 , 16 ; respectively). No significant differences were found between genotypes. **d** Normalized fEPSP amplitude in WT, $Pap^{-/-}$, and $Nt5e^{-/-}$ slices incubated with TNAP inhibitor MLS-0038949 (50 μ M) ($n = 11$ for all genotypes). There was a significant reduction in the inhibitory effect of AMP on slices from $Nt5e^{-/-}$ mice when compared to WT and $Pap^{-/-}$ (all incubated with MLS-0038949); $P < 0.005$. **e** Normalized fEPSP amplitude in WT and dKO mice incubated with MLS-0038949 ($n = 9$ and 12 ; respectively). *** $P < 0.0005$. Figure adapted from Street et al. (2013), and reprinted with permission

These results strongly suggest that AMP must first be dephosphorylated into adenosine to have physiological effects downstream of A_1R . Previous studies carried out in heterologous expression systems suggested that AMP might be able to directly activate A_1R in intact organ systems (Rittiner et al. 2012). However, these results demonstrated that adenosine is required to activate G proteins that inhibit cAMP generation (G_i) downstream signal transduction cascades that ultimately result in synaptic inhibition.

More importantly, this experiment strongly suggested that all three enzymes, PAP, NT5E, and TNAP, play a role in generating extracellular adenosine that then inhibits synaptic activity in nociceptive circuits. It also demonstrated that NT5E and TNAP contribute to the majority of adenosine generation since deletion or inhibition of both of these enzymes together resulted in reduced synaptic inhibition. However, removal of activity of all three enzymes was necessary to completely reduce the inhibitory effect of AMP application, showing that these enzymes work redundantly in nociceptive circuits of the spinal cord.

13.9 Proposed Model of Ectonucleotidase Function in Nociceptive Circuits

These studies show that TNAP, along with PAP and NT5E, plays an important role in the modulation of nociception. ATP, released by stimulated sensory neurons, activate P2X and P2Y receptors on both neurons and glia, and is thought to play a role in the transition from acute to chronic pain (Basbaum et al. 2009; Burnstock 2007; Sawynok et al. 2006; Tsuda et al. 2005; Tozaki-Saitoh et al. 2008). Ectonucleotidases then act to terminate this pronociceptive signaling by breaking down ATP, with TNAP, PAP and NT5E contributing to the final step in this breakdown, generating adenosine from AMP. Adenosine then elicits anti-nociceptive effects through A₁R. Thus, TNAP, PAP and NT5E all determine the relative extracellular purinergic tone that is either activating or inhibiting nociceptive neurons. With deletion of these enzymes, less extracellular adenosine is generated, swinging the balance in favor of excitation over inhibition.

Other studies have suggested that alterations in purinergic tone also lead to downstream changes in the levels of membrane phospholipids (Sowa et al. 2010a). For instance, activation of A₁R is thought to inhibit synaptic transmission through the activation of the G α_i subunit. However, other downstream effects of A₁R activation occur through the release and activation of the G $\beta\gamma$, which subsequently activates phospholipase C (PLC) (Jacobson and Gao 2006; Murthy and Makhoulf 1995). PLC hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP₂) in the plasma membrane leading to depletion of PIP₂. PIP₂, a membrane phospholipid, interacts with many different transmembrane proteins, including GPCRs and ion channels. Usually this interaction is necessary for physiological activation of ion channels such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) and voltage-gated calcium channels (Caterina et al. 1997, 2000; Davis et al. 2000; Rohacs et al. 2005; Sowa et al. 2010a; Suh and Hille 2005). Decreases in membrane levels of PIP₂ lead to deactivation of excitatory ion channels, resulting in an overall decrease in the excitability of neurons.

In DRG neurons, sustained activation of A₁R decreases nociceptive sensitivity by inhibiting channel activation via PIP₂ depletion (Fig. 13.3) (Sowa et al. 2010a). Since extracellular adenosine concentration is dependent on activity of

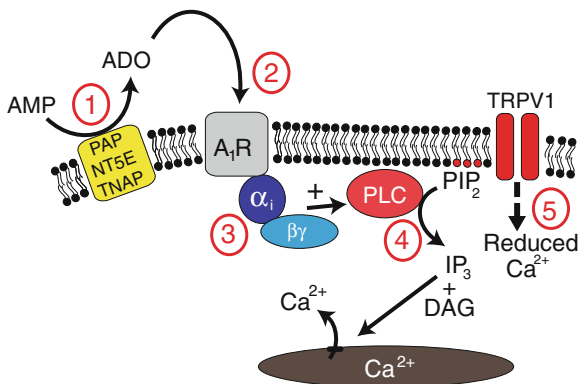


Fig. 13.3 Ectonucleotidases modulate nociceptive sensory neurons by regulating phosphoinositide tone. Proposed model showing the steps involved in ectonucleotidase-catalyzed adenosine generation, and downstream effects. 1 Extracellular AMP is hydrolyzed by TNAP, NT5E, and PAP to generate extracellular adenosine. 2 Adenosine binds and activates A₁R. 3 PLC is activated through the βγ subunit of the G_i G-protein. 4 PLC depletes PIP₂ in the plasma membrane. 5 TRPV1 channels are inhibited following PIP₂ depletion, leading to a decrease in neuronal excitability. Figure adapted from Sowa et al. (2010a) and used with permission

ectonucleotidases, these enzymes influence the levels of PIP₂ in the membrane and hence, the overall excitability of nociceptive neurons. For example, PIP₂ levels are decreased in DRG neurons following injection of PAP, while PIP₂ levels are increased in DRG from *Pap*^{-/-} and *Nt5e*^{-/-} mice (Sowa et al. 2010a). Further, the relative levels of PIP₂ in the membrane of nociceptive neurons sets the degree of sensitization that nociceptive neurons experience following nerve injury or injection of an inflammatory agent. When levels of PIP₂ are low due to increased activity of PAP, both thermal hyperalgesia and mechanical allodynia are reduced (Sowa et al. 2010a). Thus, ectonucleotidases determine activation of A₁R, which in turn determines the levels of PIP₂ in the membrane. PIP₂ then sets the overall excitability of nociceptive neurons through its interaction with ion channels and GPCRs. In fact, by directly increasing the level of PIP₂ in the plasma membrane of DRG neurons through spinal injection of PIP₂, the sensitivity of mice to noxious thermal stimuli is increased.

These findings have led to a theory that emphasizes the interaction of purinergic and cellular “phosphoinositide tone” in nociceptive circuits (Fig. 13.3). With increases in adenosine generation by ectonucleotidases, there is increased A₁R activation and increased depletion of PIP₂. This leads to decreases in synaptic transmission and membrane excitability. However, increases of extracellular ATP and ADP lead to increases in membrane excitability and neuronal sensitization. In this way, the balance of extracellular purinergic tone and membrane PIP₂ levels control the overall excitability and sensitivity of nociceptive circuits. Thus, TNAP, PAP and NT5E play critical roles in determining the overall sensitivity of these circuits.

13.10 Possible Reasons for Redundancy in Ectonucleotidases in the Dorsal Horn

DRG and spinal cord are not the first tissues reported to have redundant expression of ectonucleotidases. For instance, NT5E and TNAP act redundantly to hydrolyze AMP in both the hippocampus and airway epithelial cells (Picher et al. 2003; Zhang et al. 2012). Further TNAP is often co-expressed with NT5E and other ectonucleotidases, suggesting that redundant expression of several different ectonucleotidases is common to many different organ systems and circuits in the central nervous system (Langer et al. 2008; Zimmermann 2006). However, triply redundant expression of acid and alkaline phosphatase, along with another ectonucleotidase, has yet to be reported in any area of the CNS. This raises the question as to why nociceptive circuits display such extensive expression of ectonucleotidases. There are several possibilities that might explain this redundant expression of ectonucleotidases.

13.10.1 Importance of Nucleotide Hydrolysis

The most obvious answer lies in the proposed model linking ectonucleotidases to the control of phosphoinositide tone, which is an important determinant of neuronal sensitivity. If the build-up of extracellular nucleotides leads to sensitization of sensory neurons and the availability of adenosine keeps nociceptive neurons from becoming overly excitable, it follows that the enzymes responsible for this baseline balance have several levels of redundancy. The evidence discussed here clearly shows that loss of just one, or even two of these enzymes does not result in the complete loss of AMP hydrolysis. Therefore, some ability to maintain physiological levels of PIP₂ remains after loss of one or two ectonucleotidases.

Interestingly, some aspects of the knockout mice behavioral data support this conclusion. While both single knockouts (*Pap*^{-/-} or *Nt5e*^{-/-}) and the dKO showed increased sensitization in models of chronic inflammatory and neuropathic pain, almost all of the baseline nociceptive behavior was unaffected. Thus, it was not until an inflammatory insult was given to the animal that nociceptive behavior changed (Sowa et al. 2010b; Street et al. 2011; Zylka et al. 2008). This suggests that having at least one remaining enzyme is sufficient to maintain baseline extracellular adenosine levels and phosphoinositide tone. Unfortunately, developing a triple-knockout mouse where TNAP, PAP and NT5E are all deleted would be quite difficult considering that *Tnap*^{-/-} mice die a few weeks after birth. It could be possible to develop a neuron-specific *Tnap*^{-/-} mouse that can then be crossbred with dKO mice, but even this model might not sufficiently reduce TNAP activity since the cellular localization of TNAP in spinal circuits is unknown. However, any *Tnap*^{-/-} mouse model of nociception would also be confounded by the role TNAP plays in somatosensory circuit development. Lumbar nerve roots, used in many mouse models of nociception, are smaller in *Tnap*^{-/-} mice (Narisawa et al. 1997).

13.10.2 pH Specificity

Another possible need for the redundancy of TNAP, PAP and NT5E could be explained by the differences between these enzymes. While all three ectonucleotidases are capable of hydrolyzing AMP, these enzymes have very different functional profiles. NT5E is an exclusive 5'-monophosphatase that selectively hydrolyzes 5'-AMP in the low micromolar range and at optimal neutral pH range. PAP, on the other hand, is a promiscuous phosphomonoesterase that can also degrade 5'-nucleoside monophosphates, and a broader pH range (3–8), while TNAP dephosphorylates many extracellular substrates including ATP, ADP, and AMP at neutral and alkaline pH (Ciancaglini et al. 2010; Scheibe et al. 2000).

These differences in activity at varying pH values are especially important in the nervous system, where repeated neuronal stimulation can produce slightly acidic conditions (DeVries 2001; Wemmie et al. 2008). Also, following tissue injury, an “inflammatory soup” arises that contains protons and nucleotides (Julius and Basbaum 2001). In these settings PAP, which is more active at acidic pH may be more capable than NT5E or TNAP at degrading AMP to adenosine. Other studies have also shown that electrical nerve stimulation produced acid-alkaline, biphasic, and alkaline-acid-alkaline triphasic swings in extracellular pH in the spinal cord dorsal horn (Sykova and Svoboda 1990). These studies suggest that the changes in extracellular pH at varying levels of sensory input might necessitate the expression of an acid and an alkaline phosphatase, along with the dominant phosphatase that functions at neutral pH.

13.10.3 Substrate Specificity

An additional reason for redundancy might reflect the fact that PAP and TNAP both degrade a wide variety of substrates while NT5E is restricted to only 5'-nucleoside monophosphates. For instance, PAP can degrade another pro-nociceptive compound lysophosphatidic acid (LPA) (Tanaka et al. 2004). LPA is released from platelets and neurons following tissue injury and produces prolonged hyperalgesia and allodynia following activation of central LPA receptors (Inoue et al. 2004; Moolenaar et al. 1997). PAP also has been implicated in acting on cellular substrates that inhibit cell growth and replication (Lin et al. 1992, 1994, 2001; Quintero et al. 2007; Hurt et al. 2012). Therefore PAP might be expressed to perform other roles in addition to its role as an acid phosphatase in DRG and spinal cord.

Neuronal TNAP, in contrast, is most well known for its role in pyridoxal 5'-phosphate metabolism, which is an essential co-factor in GABA synthesis (Narisawa et al. 1997; Waymire et al. 1995). Further, TNAP is not only capable of AMP hydrolysis, but also degradation of ATP and ADP (Ciancaglini et al. 2010). Hence, TNAP might be important in the upstream hydrolysis of extracellular nucleotides as well as AMP hydrolysis. Other ectonucleotidases that degrade ATP

and ADP such as nucleoside triphosphate diphosphohydrolases 1–3 (ENTPD1–3) are expressed in DRG neurons, but ENTPD3 activity seems to dominate in nociceptive circuits (Vongtau et al. 2011). Thus, in addition to its primary role in the CNS, TNAP might also serve as a redundant ectonucleotidase that can help out in times of pH shift or large increases in extracellular nucleotides.

13.10.4 Ectonucleotidase Localization

The functional redundancy of TNAP, PAP and NT5E is dependent on the localization of each of these enzymes. While it is known that PAP and NT5E are co-expressed in some DRG neurons, their expression is found mainly in non-peptidergic neurons and some peptidergic neurons (Sowa et al. 2010b; Zylka et al. 2008). TNAP, on the other hand, is broadly expressed in DRG neurons regardless of size (Street et al. 2013). Thus, there might be different patterns of redundancy depending on the precise primary sensory afferent and its target.

Further, the precise subcellular location of TNAP, PAP and NT5E activity has yet to be determined, leaving many questions unanswered. For instance, are these effects occurring in the peripheral terminals of primary afferents, in DRG cell bodies, in primary afferent terminals in the dorsal spinal cord, or on post-synaptic terminals of dorsal horn neurons? Further, are each of these enzymes expressed in the synapse or are some enzymes also expressed on other membrane areas of neurons and glia to deal with extra-synaptic ATP release? One study placed TNAP localization directly in axonal and dendritic processes, including the synaptic cleft, arguing for a role of TNAP in synaptic transmission (Fonta et al. 2004). A₁R has also been hypothesized to be localized to pre- and post-synaptic membranes in the spinal cord (Lao et al. 2001; Li and Perl 1994; Schulte et al. 2003). It is possible that, depending on the subcellular localization of each of these enzymes and A₁R, they are not redundant at all, but rather all working in their own subcellular microdomains. None of the experiments in the spinal cord to date are able to provide the resolution to identify the precise location where TNAP, PAP and NT5E exert their effects. Until the precise location of these enzymes are known, it is impossible to identify their exact role in the modulation and regulation of nociceptive information.

13.11 Use of TNAP and Other Ectonucleotidases to Treat Pain in Humans

While administration of adenosine and A₁R agonists reduce pain, they also cause paralysis and other dangerous side effects when used to treat pain in humans (Zylka 2011). In contrast, PAP and NT5E, when injected intrathecally in mice, did not

produce motor side effects, and caused significant and long-lasting reductions in nociceptive behavior (Sowa et al. 2009, 2010b, c; Zylka et al. 2008). Further, PAP, when injected peripherally into the popliteal fossa of mice, also produced a potent and long-lasting anti-nociception without any visible side effects (Hurt and Zylka 2012). This is likely due to the fact that as enzymes, ectonucleotidases are restricted in their activity by the abundance of their substrate, namely AMP. It seems likely that all three enzymes are producing adenosine throughout the spinal cord, but while that amount made in the dorsal horn is sufficient to mediate antinociception, the amount made in the ventral horn is not enough to inhibit motor function due to the lower endogenous levels of AMP. Thus, this catalytic restriction may limit the amount of A₁R activation when these ectonucleotidases are administered, but this limitation could be advantageous by avoiding unwanted side effects.

Because of the ability to manipulate endogenous levels of nucleotides and thereby limit the level of A₁R activation both in size and in scope, TNAP, PAP and NT5E could prove to be important approaches for the prevention and treatment of chronic pain. Clearly these studies are only the very first steps in the development of new therapies. However, recombinant TNAP has been used to successfully treat rickets in patients with hypophosphatasia, a condition resulting from mutations in the TNAP gene, without adverse side effects (Whyte et al. 2012). This recombinant protein featured a bone-homing fusion tag to target the protein to the correct tissue (Millán et al. 2008). Thus, this study provides evidence of the therapeutic potential of enzyme replacement in humans to treat a chronic disorder (cf Chap. 15). In time, it will be very important to assess the feasibility and safety of using TNAP, PAP, and NT5E recombinant proteins to treat chronic pain in humans. However, in the meantime, the identification of the importance of ectonucleotidases such as TNAP has provided another target for future treatments for chronic pain.

13.12 Summary

Continued research into the role of TNAP in nociceptive circuits will enable further understanding of the exact role of TNAP in pain control. To date, studies have only focused on the expression and function of TNAP in spinal nociceptive circuits. However, there are also nuclei in the brainstem, thalamus, insula and cerebral cortex that also play major roles in the perception of pain. TNAP is broadly expressed in many areas of the brain, including the primary somatosensory cortex (Fonta et al. 2004), so it will be important to look at the possible role TNAP could play in higher processing of nociceptive signals and the development of chronic pain. It is also possible that TNAP has additional functional roles in the somatosensory system outside of its ectonucleotidase activity. The identification of TNAP and its important function in spinal nociceptive circuits has opened up many new possibilities for both the study and eventual treatment of pain.

Conflict of Interest The authors declare no conflict of interest.

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