CHARACTERIZATION OF ECTONUCLEOTIDASES IN NOCICEPTIVE CIRCUITS

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ABSTRACT

Nathaniel Adam Sowa: Characterization of ectonucleotidases in nociceptive circuits
(Under the direction of Mark J. Zylka, Ph.D.)

Pain is one of the most common medical complaints in the United States, affecting almost a quarter of American adults. There are numerous treatments for acute and chronic pain, but none of them are completely effective and many have intolerable side effects. New treatments are needed that are safer, more efficacious, and more cost-effective. We have focused on trying to understand better the mechanisms involved in the regulation of pain (nociceptive) signaling in order to develop novel therapies. Two important compounds involved in pain signaling are adenosine triphosphate (ATP) and adenosine. ATP has pro-nociceptive properties, while adenosine is antinociceptive. ATP can be converted to adenosine through a step-wise process catalyzed by enzymes on the surface of cells called ectonucleotidases. These enzymes could play a pivotal role in regulation of nociception by degrading pro-nociceptive ATP while simultaneously producing antinociceptive adenosine. Prior to this work, the exact ectonucleotidases present in nociceptive circuits were unknown.

Here, we identify and characterize the first two known AMP-degrading ectonucleotidases involved in nociception, prostatic acid phosphatase (PAP) and Ecto-5'-nucleotidase (NT5E). Genetic deletion of these enzymes does not affect acute nociception, but leads to enhanced pain sensitivity in chronic inflammatory and neuropathic pain models. Conversely, intraspinal injection of PAP or NT5E protein has antinociceptive, antihyperalgesic, and antiallodynic effects that last longer than the opioid analgesic

morphine. Both PAP and NT5E suppress pain by the production of adenosine from endogenous AMP and subsequent activation of the A_1-adenosine receptor (A_1R). Further, chronic activation of A_1R by PAP leads to depletion of cellular levels of the signaling molecule PIP_2. Depletion of PIP_2 before or after chemical or physical injury (through injection of PAP) reduces pain hypersensitivity, highlighting an important role for PIP_2 levels in the modulation of nociceptive signaling. We are the first to show this important role for PIP_2 in setting the dynamic pain threshold in nociceptors. These studies not only identify two potentially new targets for the development of chronic pain therapy, but also highlight a new model for the dynamic modulation of pain sensitivity through the regulation of neuronal PIP_2 levels.

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A_1R	A1 adenosine receptor
AD	Adenosine deaminase
ADP	Adenosine diphosphate
AK	Adenosine kinase
AMP	Adenosine monophosphate
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
BK	Bradykinin
bPAP	Bovine prostatic acid phosphate
Ca^{2+}	Calcium
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CPA	N^6-cyclopentyladenosine
CPX	8-cyclopentyl-1,3-dipropylxanthine
DAG	Diacylglycerol
DRG	Dorsal root ganglion (ganglia)
E-NPPase	Ectonucleotide pyrophosphatase / phosphodiesterase
E-NTPDase	Ectonucleoside triphosphate diphosphohydrolase
FRAP	Fluoride resistant acid phosphatase
GPCR	G-protein coupled receptor

hPAP	Human prostatic acid phosphatase
IB4	Isolectin B4
IP_3	Inositol triphosphate
ITU	5-iodotubercidin
LPA	Lysophosphatidic acid
mPAP	Mouse prostatic acid phosphatase
Mrgprd	Mas-related G-protein coupled receptor D
mNT5E	Mouse recombinant ecto-5’-nucleotidase
NGF	Nerve growth factor
NT5E	Ecto-5’-nucleotidase
PAP	Prostatic acid phosphatase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIPK	Phosphatidylinositol-4-phosphate-5-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
S-hPAP	Secretory human prostatic acid phosphatase
SNI	Spared nerve injury
Thr	Thrombin
TMP	Thiamine monophosphate
TMPase	Thiamine monophosphatase
TM-PAP	Transmembrane prostatic acid phosphatase

TNAP	Tissue non-specific alkaline phosphatase
TRPM8	Transient receptor potential cation channel M8
TRPA1	Transient receptor potential cation channel A1
TRPV1	Transient receptor potential cation channel V1
WT	Wild type

CHAPTER 1

Introduction and Background

1.1) The Burden of Chronic Pain

Pain – be it physical or emotional – is one of the most common medical complaints in the United States. In a recent survey, over one-quarter (26%) of all adults in the U.S. reported that they suffered from pain (of any type) that persisted for greater than 24 hours during the month prior to interview (2006). Almost one-half of these individuals (42%) reported their pain had lasted for greater than 1 year. Chronic pain can be extremely debilitating, affecting physical and mental well-being and profoundly diminishing quality of life. Patients reporting moderate to severe chronic pain describe difficulty performing everyday activities, including exercising, sleeping, doing chores, participating in social activities, working, and caring for children (1999). Moderate to severe chronic pain sufferers also report difficulty maintaining attention and focusing on important tasks, as well as emotional difficulties, including listlessness, feelings of worthlessness, anxiety, and depression (1999). All of these problems significantly worsen when pain is inadequately controlled.

The treatment of pain is extremely expensive. The direct costs of pain treatment, including medical practitioner and hospital visits, drugs, and therapies are estimated to be over $100US billion annually (2006; Luo et al., 2004). These costs do not take into account other societal costs, including loss of work time and productivity. Together, these make pain

one of the most costly human conditions. Despite these impacts on personal and societal well-being, pain remains largely undertreated and poorly understood. These factors led the U.S. Congress to declare the decade starting in 2001 as the "Decade of Pain Control and Research." (Title VI, Sec. 1603, of H.R. 3244).

Treatments for pain – both scientifically vetted and otherwise – abound. Pharmaceutical treatments are the most common, and include non-steroidal anti-inflammatory drugs (NSAIDS; aspirin, ibuprofen, acetaminophen, naproxen), COX inhibitors (celecoxib, rofecoxib), opioids (morphine, codeine, oxycodone, fentanyl), antidepressants, anticonvulsants, and specific ion channel blockers. While they are some of the most commonly prescribed drugs on the market, pain relievers also carry significant side effects that many patients cannot tolerate, making other modes of therapy necessary. Other treatments include injections of local anesthetics (lidocaine), nerve blocks, physical therapy, electrical stimulation, psychological therapy, surgery, and acupuncture. In addition, chronic pain patients are constantly bombarded with advertisements for alternative therapies, homeopathic remedies, and natural supplements which claim to treat pain. This menagerie of treatments can be very confusing, expensive, and time consuming for patients, further raising the burden of disease. Further, no single treatment is often sufficient for many patients, leading to frustration, anger, and additional emotional challenges that often worsen the pain symptoms. Thus, while many modes of "pain relief" are available to patients, many still suffer, requiring further advances in pain therapy.

It is with this knowledge in mind that we have set out to better understand the nociceptive (pain-sensing) nervous system, in order to design better treatments for both acute and chronic pain. We have focused on understanding the endogenous mechanisms present in

the nociceptive nervous system that modulate signaling in order to identify new targets for therapy. We hope that these studies provide increased knowledge of pain signaling and pathology and will also lead to novel therapeutic approaches which are so desperately needed by millions of people.

1.2) The Nociceptive Nervous System

Every moment, the body receives a wealth of information about its surrounding environment. The task of detecting, integrating, and transmitting this information belongs to the peripheral sensory nervous system. Highly specialized nerve fibers provide information to the central nervous system about the environment and the state of the organism. In the skin alone, there are fibers that detect cooling, warmth, vibration, pressure, noxious chemicals and touch. Some fibers can detect several of these stimuli (polymodal), while others are specialized for one particular type of input (Julius and Basbaum, 2001; Meyer et al., 2006). Within these groups, there are fibers belonging to neurons that are specialized in the detection of noxious (injurious or potentially injurious) stimuli, which are called nociceptors (Sherrington, 1906). These fibers have been shown, using electrophysiological studies, to be excited by noxious heat, intense pressure, and chemical irritants, but not by innocuous warming or light touch (Burgess and Perl, 1967). It is these neurons that are responsible for protecting organisms from potentially harmful stimuli in their environment.

Fibers innervating the head and body have their cell bodies in the trigeminal and dorsal root ganglia (DRG), respectively, which lie in and along the dorsal regions of the brainstem and spinal cord. These pseudo-unipolar neurons have a very long axon that branches into two distinct processes (Meyer et al., 2006). The distal process extends into the

periphery to detect and relay information from external stimuli, while the proximal process extends into the central nervous system, synapsing with neurons in the spinal cord or brainstem. Information is then carried to higher levels of the CNS for integration and interpretation.

There are three broad classes of sensory neurons found in the trigeminal ganglia and DRG that supply the skin (Figure 1.1). Large diameter neurons give rise to relatively large diameter, myelinated Aβ fibers. These fibers conduct action potentials very rapidly, and are largely involved in the detection of innocuous stimuli applied to skin, muscles, and joints (Julius and Basbaum, 2001). Slightly smaller in diameter are the thinly myelinated Aδ fibers. These fibers transmit action potentials more slowly than Aβs and their activation is thought to lead to pricking, sharp, and perhaps aching pain (Julius and Basbaum, 2001; Meyer et al., 2006). There are two broad classes of Aδ nociceptors (Type I and Type II) that differ in their thermal thresholds, ability to respond to mechanical stimuli, and their functional firing properties (Dubner and Hu, 1977; Meyer et al., 2006; Treede et al., 1998). These fibers are thought to play some role in detection of noxious heat and in the sensitization process following burn or chemical injury (Campbell et al., 1979; Ringkamp et al., 2001; Treede et al., 1998). The smallest diameter DRG neurons give rise to small, unmyelinated C fibers. C-fiber nociceptors are mostly polymodal, responding to noxious thermal, mechanical, and chemical stimuli (Julius and Basbaum, 2001; Meyer et al., 2006). They propagate action potentials rather slowly, and are thought to give rise to a burning pain sensation when activated. C fibers have slowly adapting responses to mechanical and thermal stimuli, and are heavily involved in the sensitization (hyperalgesia) process following tissue injury (Meyer et al., 2006; Peng et al., 2003).

1.3) Classification of Nociceptors Based on Molecular Markers

In addition to their general size and firing properties, nociceptors can be further characterized by particular cellular markers that allow for study of their peripheral and central projections. These markers include cell surface receptors, peptides stored and released by the cells, and enzymes (Meyer et al., 2006). They often differ in their relative abundance between species (Zwick et al., 2002) and can change following inflammation or nerve injury (Meyer et al., 2006), but can be extremely useful in understanding general properties about classes of neurons and can even highlight functional and anatomically distinct circuits (Cavanaugh et al., 2009; Zylka et al., 2005). Large-diameter DRG neurons (Aβ and some myelinated Aδ) are uniformly labeled with antibodies against the phosphorylated heavy chain neurofilament protein NF200. This marker can easily distinguish between these larger sensory neurons and small DRG cells, for which there are many more markers.

Small DRG neurons are broadly subdivided into peptidergic and nonpeptidergic neurons (Figure 1.2) (Julius and Basbaum, 2001; Meyer et al., 2006; Zylka, 2005). Peptidergic neurons contain neuropeptides, such as substance P, calcitonin gene-related protein (CGRP), and somatostatin. These neurons also express TrkA, the high-affinity tyrosine kinase receptor for nerve growth factor (NGF) and depend upon NGF-TrkA signaling for survival during development (Fitzgerald, 2005). Studies examining substance P or CGRP expression in rat have shown that about 40% of all DRG cells – 50% of C fibers, and 20% of Aδ fibers – are classified as peptidergic (Lawson et al., 1996; McCarthy and Lawson, 1989). Peptidergic neurons project specifically to lamiae I and II_{outer} in the dorsal

horn of the spinal cord (Hunt and Mantyh, 2001; Zylka, 2005), and they extend their axons into visceral, dermal, and epidermal targets (Perry and Lawson, 1998; Zylka et al., 2005).

Nonpeptidergic neurons contain fluoride resistant acid phosphatase (FRAP), bind the plant lectin IB4 from *Griffonia simplicifolia*, and express the ATP-gated ion channel $P2X_3$ (Meyer et al., 2006; Silverman and Kruger, 1988a; Silverman and Kruger, 1988b; Snider and McMahon, 1998; Vulchanova et al., 1998). Nonpeptidergic nociceptors lose expression of TrkA during development and rely on signaling through the receptor tyrosine kinase Ret for survival (Fitzgerald, 2005). While staining for IB4 or FRAP has traditionally been used to discriminate nonpeptidergic neurons from peptidergic cells, it should be noted that there is some colocalization of both of these markers with substance P and/or CGRP in DRG (Bergman et al., 1999; Carr et al., 1990; Wang et al., 1994). Nonpeptidergic neurons project specifically to lamina II_{inner} in the dorsal horn and extend their axons primarily to the skin, with some afferents projecting to muscle (Perry and Lawson, 1998; Zylka, 2005).

While these classifications are not necessarily functional in nature, they do provide some insight into broad classes of nociceptors. For example, the remarkable segregation of peptidergic and nonpeptidergic axon terminals in the dorsal horn hints at a unique central connectivity between these classes. In addition, peptidergic and nonpeptidergic neurons can also have different morphological and anatomical characteristics within a given tissue type, hinting at conserved functional differences in these regions (Zylka et al., 2005). Further, certain receptors involved in signal transduction also segregate between peptidergic and nonpeptidergic nociceptors. For example, the pro-nociceptive ATP receptor $P2X_3$ is found almost exclusively in $IB4^+$ neurons, while the noxious heat and capsaicin receptor TRVP1 is expressed primarily in peptidergic neurons in the mouse. These segregations hint at

functional differences between these nociceptor classes and can be useful in understanding expression patterns and possible functions of newly discovered proteins and markers.

1.4) Important Transducers and Modulators of Nociception

Understanding the properties of nociception requires a basic knowledge of the important players in the detection and production of nociceptive signals from afferent neurons. Here I will outline, briefly in some cases and in detail in others, the mechanisms of noxious stimuli detection and the important channels and receptors involved in the creation and modulation of nociceptive neurotransmission.

1.4.1) Detection of Mechanical Stimuli

Nociceptors are capable of detecting noxious mechanical stimuli due to direct pressure, tissue deformation or osmotic stress. While the properties of nociceptor responses to these stimuli have been intensely studied (Meyer et al., 2006), surprisingly, the identification of the direct mechanotransducers present in mammals remains elusive. Candidates have been identified from invertebrate systems, but studies on mammalian orthologs have been inconclusive.

Studies from the worm *Caenorhabditis elegans* have shown members of the DEG/ENaC ion channel family to be important in the detection of gentle touch (Julius and McCleskey, 2006). Mammalian homologs of these channels (ASIC1, ASIC2, and ASIC3) are expressed in primary sensory neurons (Garcia-Anoveros et al., 2001), but targeted deletions of these genes produce only subtle alterations in mechanosensitivity in mice,

questioning their role in mechanotransduction (Price et al., 2000; Price et al., 2001; Roza et al., 2004).

Studies from yeast, flies, and worms have also implicated members of the transient receptor potential (TRP) family of ion channels in the detection of mechanical stimuli. However, their role in mammalian mechanotransduction remains unclear (Christensen and Corey, 2007). TRPA1 is thought to be involved in acute noxious mechanosensation and cold sensation. However, mice null for TRPA1 show normal mechanical sensitivity to most stimuli, with only a deficit in the detection of intense stimuli at high g-forces (Kwan, 2006; Petrus et al., 2007). In addition, while antagonists of TRPA1 decrease nociceptor responses to mechanical stimuli in vitro, application in vivo does not affect acute mechanosensation (Kerstein et al., 2009; Petrus et al., 2007). Thus, while TRPA1 likely plays a role in mechanosensation, possibly through indirect activation due to release of reactive compounds from damaged tissue or increases in intracellular calcium, there is no evidence for direct activation of the channel by mechanical stimuli (Christensen and Corey, 2007; Hinman et al., 2006; Macpherson et al., 2007; Zurborg et al., 2007). Another TRP channel, TRPV4, is activated in vitro by changes in osmolarity (Liedtke et al., 2000; Strotmann et al., 2000). However, targeted disruption of TRPV4 in mice leads to only minor changes in mechanosensory responses, suggesting it may not be important in mechanotransduction in vivo (Liedtke et al., 2000; Mizuno et al., 2003; Suzuki et al., 2003).

1.4.2) Detection of Thermal Stimuli

1.4.2.1) Detection of Cold

Exposure to noxious cold temperatures (< 4ºC) leads to intense, burning pain. Activation of nociceptors by noxious cold has been proposed to occur through a number of mechanisms, including activation of Ca^{2+}- and/or Na^{2+}-permeable channels, inhibition of background K^+ channels, inhibition of Na^+/K^+-ATPases, or differential effects of cold on voltage-gated Na^+ or K^+ conductances (Julius and McCleskey, 2006). Recent work has shown the potential for specific excitatory "cold receptors." Most prominent among these is the TRP channel family member TRPM8, which is gated by both menthol (the ingredient responsible for the "cool" sensation in gum, cigarettes, and other products) and cold (McKemy et al., 2002). TRPM8 is activated by both cool (26ºC-15ºC) and cold (<15ºC) temperatures in vitro and is responsible for detection of cool temperatures in vivo. Interestingly, while TRPM8 is upregulated following neuropathic pain (Frederick et al., 2007; Proudfoot et al., 2006), its role in mediating cold, mechanical, or thermal hypersensitivity under pathophysiological conditions is unclear. In fact, TRMP8 agonists can actually attenuate thermal and mechanical hypersensitivity in some models of neuropathic pain, highlighting a possible role for the channel in cold-mediated analgesia (Proudfoot et al., 2006; Xing et al., 2008). Mice null for TRPM8 still retain an aversion to intense cold, suggesting TRPM8 is responsible for detection of only cool and some noxious cold temperatures in vivo (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Thus, other mechanisms must be responsible for detection of extreme cold temperatures.

Another candidate for a noxious cold sensor is the aforementioned TRPA1. When heterologously expressed in CHO cells, TRPA1 is activated by cold temperatures with a threshold that is lower than TRPM8 (Bandell et al., 2004b; Sawada et al., 2007; Story et al., 2003c). However, the role of TRPA1 in detection of noxious cold is controversial due to

discrepancies in cold activation in different heterologous expression systems (Jordt et al., 2004; Nagata et al., 2005), the possibility of indirect channel activation due to rises in intracellular Ca^{2+} release (Zurborg et al., 2007), a lack of correlation between responses of somatosensory neurons to both mustard oil (a known TRPA1 agonist) and cold (Babes et al., 2004; Bautista et al., 2006b; Jordt et al., 2004), and conflicting reports of cold-sensing deficits in TRPA1 null mice (Bautista et al., 2006b; Kwan et al., 2006). While a recent study attempted to address these concerns, showing direct cold-activation of TRPA1 in vitro, expression of the channel in a distinct subset of cold-sensitive primary sensory neurons, and a distinct noxious cold-sensing deficit in TRPA1 null mice (Karashima et al., 2009), the controversy over TRPA1's role in noxious cold sensation is likely to continue.

1.4.2.2) Detection of Heat

In cell culture, approximately 45% of small- and medium-diameter DRG neurons show heat-evoked membrane currents that initiate at an activation threshold of ~42ºC (Julius and McCleskey, 2006; Nagy and Rang, 1999). Based on size and heat responsiveness, these are likely C-fiber and Type II Aδ-fiber nociceptors. In addition, about 5% of the medium- to large-diameter Type I Aδ fiber DRG neurons respond to heat at the higher activation threshold of ~51ºC (Cesare et al., 1999b; Nagy and Rang, 1999). These different properties suggest a different mechanism of thermosensation and activation. It is now clear that the thermal sensitivity of the smaller, C-fiber and Type II Aδ-fiber nociceptors is due to the expression of the capsaicin and noxious thermosensor TRPV1. Since TRPV1 plays an important role in the following studies, I will spend some time describing the characteristics of this important channel.

1.4.2.2.1) TRPV1

TRPV1 is a nonselective cation channel that responds to noxious heat at temperatures above 43ºC, similar to the properties seen in small- and medium-diameter DRG neurons in culture (Caterina et al., 1997). Fittingly, TRPV1 is expressed predominantly in a subset of small- and medium-diameter DRG neurons in mice (Pingle et al., 2007). The channel has equal selectivity for the passage of most univalent cations, and is moderately selective for divalent cations (Caterina et al., 1997). In addition, it is highly permeable to protons, large polyvalent cations, polyamines, and several organic cationic dyes (Ahern et al., 2006; Meyers et al., 2003). The relative permeability and large pore size of the channel has allowed it to be used as a tool to deliver small-molecule anesthetics (Binshtok et al., 2007; Binshtok et al., 2009).

Activation of the channel by heat shows a steep temperature dependence (Liu et al., 2003), and increases in temperatures in the subthreshold range (< 43ºC) can synergistically enhance currents produced by other TRPV1 agonists (Babes et al., 2002). The exact mechanism underlying heat activation of the channel is unclear, but is likely due to changes in intrinsic voltage-sensitivity of the channel as a result of changes in temperature (Nilius et al., 2005). What is clear is that cells heterologously expressing TRPV1, as well as $TRPV1^{+}$ DRG neurons, show heat-evoked single-channel currents, indicating TRPV1 is an intrinsically heat-activated channel that underlies the native “moderate” threshold thermosensation seen in C- and Aδ-fiber nociceptors (Caterina and Julius, 2001).

In addition to heat, other stimuli can activate the channel. TRPV1 was initially cloned based on its ability to respond to capsaicin, the pungent ingredient found in chili

peppers (Caterina et al., 1997). Activation by capsaicin and other vanilloids (including resiniferatoxin and olvanil) occurs following binding at intracellular regions of the channel (Pingle et al., 2007) and has similar characteristics to activation with noxious heat, explaining why ingestion or exposure to these compounds leads to an intense burning sensation. Other compounds similar in structure to vanilloids can also activate the channel, including the endocannabinoid anandamide, oleoyl-dopamine, the lipoxygenase product 12-HPETE, and *N*-acetylethanolamines (Ahern, 2003; Pingle et al., 2007; Ross, 2003). In addition, TRPV1 is activated by allicin (the pungent compound in garlic) (Macpherson et al., 2005; Macpherson et al., 2006), nitric oxide (Yoshida et al., 2006), camphor (Xu et al., 2005), spider toxins (Siemens et al., 2006), and is potentiated by ethanol (Trevisani et al., 2002).

Extracellular protons and cations can also activate or sensitize the receptor. The channel is activated directly at pH < 6 and sensitized at pH 6 – 7 or by the presence of high levels of extracellular Na^{+}, Mg^{2+}, or Ca^{2+} (Ahern et al., 2005; Tominaga et al., 1998). This is due to electrostatic interactions with positively charged extracellular domains (Jordt et al., 2000; Welch et al., 2000). Interestingly, responses to cations or protons are greater following TRPV1 sensitization, suggesting they play a role in inflammatory pain signaling (Ahern et al., 2005).

Sensitization of TRPV1

Numerous stimuli can lead to sensitization of TRPV1. The release of inflammatory mediators (prostaglandin E_2, glutamate, bradykinin, nerve growth factor (NGF), and ATP) following tissue injury or inflammation can indirectly sensitize the channel through a variety of mechanisms (Levine and Alessandri-Haber, 2007). This sensitization leads to activation

of the channel at much reduced thermal thresholds, leading to the thermal hyperalgesia experienced following injury (Liang et al., 2001; Tominaga et al., 1998). Several mechanisms of sensitization have been proposed. First, NGF-receptor activation leads to downstream activation of p38 mitogen-activated protein kinase (MAPK), which in turn, increases transcription and surface expression of TRPV1 (Ji et al., 2002). NGF-receptor activation can also lead to phosphorylation of the channel by Src kinase, which increases insertion of the channel into the surface membrane (Zhang et al., 2005a).

Phosphorylation of the channel by other kinases can also sensitize TRPV1. Protein kinase C (PKC) is capable of phosphorylating several sites on TRPV1 (Huang et al., 2006c), but only two residues have been shown to affect function of the channel to date (Bhave et al., 2003; Trevisani et al., 2002). Phosphorylation by PKC increases channel activity by increasing the channel's open probability time following activation (Vellani et al., 2001), leading to increased thermal hyperalgesia in vivo (Bolcskei et al., 2005; Cesare et al., 1999a). Protein kinase A (PKA) can also phosphorylate TRPV1 at multiple amino acid residues, of which three are functionally important (Huang et al., 2006c). Although PKA activation sensitizes capsaicin- and heat-evoked currents in vitro (Lopshire and Nicol, 1998; Luciano De et al., 2001), PKA-mediated phosphorylation of the channel actually leads to decreased desensitization rather than true sensitization (Mohapatra and Nau, 2003). Regardless of the exact mechanism, the ultimate effect of PKA pathway activation is increased thermal hyperalgesia in vivo (Hucho and Levine, 2007a). Interestingly, while PKC and PKA are usually activated through different signaling pathways, activity of protease-activated receptor 2 (PAR2) , which is activated during inflammation, leads to activation of both PKC and PKA and subsequent TRPV1 sensitization (Amadesi et al., 2006).

Desensitization of TRPV1

One of the important physiologic properties of the channel is that upon activation, it undergoes desensitization. This is true whether the channel is activated by a long-lasting single application of agonist or by repeated short-term applications (tachyphylaxis) (Pingle et al., 2007). Desensitization ultimately requires the presence of intracellular Ca^{2+}, but likely involves more than one mechanism. In one model (Rohacs, 2009; Rohacs et al., 2008), Ca^{2+} that enters the cell through the stimulated channel activates the calcium-sensitive phospholipase C (PLC) (Figure 1.3). Once activated, PLC degrades its target phospholipid phosphoinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). PIP_2 activates TRPV1 through a direct interaction with the channel (see below). Thus, by depleting PIP_2, PLC activation decreases channel activity. This model is supported by several lines of evidence, including a requirement for PIP_2 for TRPV1 activity in excised patches (Klein et al., 2008; Lukacs et al., 2007; Stein et al., 2006), a dependence on the re-synthesis of PIP_2 for removal of desensitization (Liu et al., 2005), a reduction of desensitization in the presence of PLC inhibitors (Lishko et al., 2007; Lukacs et al., 2007), and the reduction of desensitization by the application of exogenous PIP_2 in whole-cell patch clamp experiments (Lishko et al., 2007; Lukacs et al., 2007). While this data is compelling, PLC inhibition or PIP_2 application are insufficient to completely block desensitization, suggesting other mechanisms are involved (Lukacs et al., 2007). Indeed, direct interaction of the Ca^{2+} sensor calmodulin is though to be involved in desensitization, as is calmodulin dependent activation of calcineurin and subsequent channel dephosphorylation (Docherty et

al., 1996; Lishko et al., 2007; Mohapatra and Nau, 2005; Numazaki et al., 2003; Rosenbaum et al., 2004).

PIP_2 regulation of TRPV1

Regulation of TRPV1 activity by PIP_2 was first proposed by Chuang and colleagues (2001), who suggested that PIP_2 tonically inhibits the channel and this inhibition is released upon PLC activation and PIP_2 hydrolysis. They later hypothesized that PIP_2 bound to TRPV1 via a polybasic region in the C-terminus of the channel (Prescott and Julius, 2003). However, the inhibitory role of PIP_2 was later refuted by electrophysiologic studies in excised patches, which showed consistent activation of TRPV1 by PIP_2, as well as by the related phosphoinositides PI(3,4,5)P and PI(3,4)P (Klein et al., 2008; Lukacs et al., 2007). In addition, as discussed above, PIP_2 has been convincingly shown to reduce desensitization, further disputing an inhibitory role for the lipid (Rohacs, 2009). How can these seemingly contradictory results be reconciled?

One model has been proposed by Rohacs and colleagues (2008), based on the stimulus intensity placed on the channel. They point out that sensitization of TRPV1 following PIP_2 depletion occurs when the channel is moderately stimulated at low capsaicin concentrations (< 1 μM). Further, the inhibitory effect of PIP_2 is not seen in studies conducted in excised patches, suggesting the effect is indirect in nature. They have shown that depletion of the lipid in whole cells leads to further activation of the channel when stimulated by moderate heat or capsaicin concentrations, suggesting a partial inhibition by PIP_2 in intact cells (Lukacs et al., 2007). They propose this may be due to either competition between inhibitory PIP_2 and a stimulatory cytosolic protein for a regulatory binding region

on the channel, or the presence of an inhibitory cytosolic protein that interacts with the channel only when bound to PIP_2 (Rohacs et al., 2008). However, with stimulation of high capsaicin concentrations, the channel is activated, not inhibited by PIP_2.

Based on these results, they proposed a dual inhibitory and activating effect at low stimulus inputs that could result in a bell-shaped dependence on PIP_2 (Figure 1.4). In this model, if resting PIP_2 concentrations are high, moderate depletion of PIP_2 results in activation of the channel, while a more severe depletion results in inhibition. At high stimulus levels, they propose a simplified model where depletion of PIP_2 leads to inhibition of the channel. It should be noted, however, that another group has shown that depletion of PIP_2 at both low and high stimulus levels leads to inhibition of TRPV1, arguing against any inhibitory role for PIP_2 (Klein et al., 2008). However, differences in experimental protocols could be responsible for the discrepancies between the studies.

Further complicating the picture is the recent discovery of the PIP_2-binding protein Pirt (Kim et al., 2008a). Pirt is expressed specifically in DRG neurons and can bind to both TRPV1 and phosphoinositides. Deletion of Pirt reduces capsaicin-evoked currents, while addition of Pirt enhances these currents in vitro. It was suggested that Pirt is required for the stimulatory effect of PIP_2 on TRPV1, but this is probably not strictly true, since even low amounts of PIP_2 can enhance TRPV1 activity in excised patches where Pirt is unlikely to be present. While Pirt may not be entirely necessary for the stimulatory effect of PIP_2 on TRPV1, it clearly plays an important role in addition to the effects of direct lipid binding to the channel.

The most telling information on the role of TRPV1 in thermosensation comes from studies on TRPV1-null mice (*Trpv1*$^{-/-}$) (Caterina et al., 2000a; Davis et al., 2000a). These

mice show reduced pain behavior at temperatures > 48ºC in hot plate and radiant heat tests, but show normal thermosensation at temperatures below 48ºC. Consistent with this, electrophysiologic studies from these mice revealed the presence of heat-sensitive C fibers, indicating that other thermosensors are present besides TRPV1. Amazingly, *Trpv1*$^{-/-}$ mice fail to develop thermal hyperalgesia following induction of inflammation by CFA, mustard oil, or pro-algesic molecules like bradykinin (Caterina et al., 2000a; Chuang et al., 2001; Davis et al., 2000a). Thus, in addition to a role in detection of noxious thermal stimuli TRPV1 plays a vital role in the production of thermal sensitization following pro-inflammatory insults in vivo. However, the behavior of *Trpv1*$^{-/-}$ mice point to the presence of additional thermosensors involved in noxious heat sensation.

1.4.2.2.2) Other Thermosensors

TRPV2, TRPV3, and TRPV4 are all responsive to warm or hot temperatures, but their roles in thermosensation are still undetermined (Levine and Alessandri-Haber, 2007). TRPV2 has a high threshold of activation (>52ºC) and is expressed in medium-diameter DRG neurons, making it a good candidate for the thermosensor in Aδ fiber nociceptors (Ahluwalia et al., 2002; Caterina et al., 1999). However, mice lacking TRPV2 have normal thermal responses, making its role unclear in vivo (Park et al., 2008). TRPV3 is activated by warm temperatures (>34ºC) and is sensitized by camphor and several natural irritants (Peier et al., 2002; Xu et al., 2006). While mice null for TRPV3 show impaired thermosensation in warm preference and tail immersion tests (Moqrich et al., 2005), TRPV3 is not expressed in the DRG in mice (Peier et al., 2002), suggesting this effect is due to channel activity elsewhere. TRPV4 is activated by innocuous heat (>27ºC), as well as numerous other stimuli

(Guler et al., 2002; Levine and Alessandri-Haber, 2007). *Trpv4*$^{-/-}$ mice do not show deficits in acute thermosensation, but develop reduced thermal hyperalgesia in response to capsaicin or carrageenan (Todaka et al., 2004). However, whether these effects are due to TRPV4 activity in sensory neurons or in keratinocytes is unclear.

1.4.3) Modulators of Nociception

While the sections above highlight specific transducers of nociceptive signals, there are numerous chemical mediators that are released upon tissue injury that can sensitize nociceptors. This sensitization leads to increased or abnormal nociceptor activity and is important in the development of hyperalgesic states seen in chronic pain. An exhaustive review of all inflammatory mediators involved in nociception is beyond the scope of this dissertation. However, Table 1.1 lists many pro-nociceptive molecules and their actions. For purposes of these studies, this dissertation will focus on the actions of adenosine and adenosine phosphates in the modulation of pain.

1.5) Adenine-containing Nucleotides and Nociception

The adenine-containing nucleotides ATP, ADP, and adenosine are important for neurotransmission and nociception (Bleehen and Keele, 1977; Burnstock, 1972). Importantly, ATP and ADP are pro-nociceptive, while adenosine is predominantly antinociceptive. These characteristics highlight the fact that ATP and ADP act through a different class of receptors (P2 receptors) than adenosine (P1 receptors) (Burnstock, 2009). Because of their prominent roles in nociception, receptors in both classes have become important therapeutic targets for acute and chronic pain. Since interconversion of these

different adenosine phosphates readily occurs in both intracellular and extracellular spaces, significant attention has also been paid to the mechanisms underlying these reactions. Control of these pathways could allow for shifting of purinergic signaling from pro-nociceptive ATP and ADP to antinociceptive adenosine in states of hyperalgesia. Below, I will highlight the roles of these compounds in nociception and discuss the enzymes involved in their interconversion in the nervous system.

1.5.1) ATP and ADP in Nociception

Early studies with ATP established it as a potential initiator of pain when applied onto skin or infused into muscle (Bleehen and Keele, 1977; Collier et al., 1966; Mork et al., 2003). In addition, ATP potentiated pain sensation due to other pro-nociceptive stimuli, such as capsaicin or ultraviolet light (Hamilton et al., 2001; Tsuda et al., 2000). Since then, the role of ATP as a pro-nociceptive molecule has been confirmed in numerous animal models of acute, inflammatory, and neuropathic pain (see Wirkner et al., 2007). From these studies a unifying hypothesis of the role of purinergic signaling in the initiation of pain has emerged, which has been expanded in recent papers (see Burnstock, 2009).

ATP and ADP exert their effects through P2 receptors, of which there are two families – P2X and P2Y. P2X receptors are ligand-gated, cation-selective channels that are permeable to Na^+ and Ca^{2+} (Sawynok, 2007). There are seven known P2X receptor subunits currently identified ($P2X_{1-7}$) (Khakh et al., 2001). Of these, only four ($P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$) are thought to be involved in nociception (Sawynok, 2007). P2Y receptors are metabotropic G-protein coupled receptors whose stimulation leads to activation of various signaling pathways, depending on the specific receptor type (Burnstock, 2009). There are

eight known P2Y receptors, of which only four ($P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$) have been potentially implicated in nociception (Wirkner et al., 2007). While P2X receptors respond predominantly to ATP or ATP analogs, some P2Y receptors respond to both pyrimidine and purine nucleosides (Burnstock, 2009). In addition, some P2Y receptors are preferentially activated by nucleoside diphosphates (Burnstock, 2009). As a result of their different agonists and signaling properties, P2X and P2Y receptors have different roles in nociception.

1.5.1.1) P2X receptors

While all seven cloned mammalian P2X receptors are present in sensory ganglia, only three have been implicated in the regulation of nociception (Khakh, 2001). Of these, $P2X_3$ is the most prominent. Approximately 40% of cultured rat DRG neurons show immunoreactivity for $P2X_3$, and the vast majority of these cells also express IB4 (Bradbury et al., 1998; Vulchanova et al., 1997). $P2X_3$ colocalizes extensively with other markers of nonpeptidergic neurons, including *MrgprD* and FRAP (Zylka et al., 2005; Zylka et al., 2008). $P2X_3^+$ neurons project to inner lamina II in the dorsal horn of the spinal cord. In addition to forming homotrimeric receptors, $P2X_3$ can also form heterotrimeric receptors with $P2X_2$, which is also expressed in some DRG neurons (Wirkner et al., 2007). While $P2X_{2/3}$ heteromeric receptors play a role in nociception, they are not necessary for mediating acute pain responses, as $P2X_2$ null mice do not show changes in acute nocifensive behavior (Cockayne et al., 2005). There is no evidence that homomeric $P2X_2$ receptors play a role in nociception.

Numerous studies support a role for $P2X_3$ and $P2X_{2/3}$ receptors in acute, inflammatory, neuropathic, visceral, and migraine pain (see Wirkner et al., 2007 for review).

Highlights of these studies include the following. Administration of ATP or the $P2X_3$ receptor agonist αβ-methylene ATP (αβ-MeATP) excites C-fibers in vitro and in vivo (Dowd et al., 1998b; Hamilton et al., 2001; Hilliges et al., 2002). Peripheral administration of ATP and αβ-MeATP induces spontaneous pain behaviors, thermal hyperalgesia, and mechanical allodynia, which are enhanced by inflammation (Hamilton et al., 1999; Sawynok and Reid, 1997; Tsuda et al., 2000). This is not surprising, since inflammation leads to upregulation of $P2X_3$ expression and sensitizes the receptor to ATP, possibly through phosphorylation (Dai et al., 2004a; Paukert et al., 2001; Xu and Huang, 2002). This is particularly important because inflammation leads to elevated levels of extracellular ATP (Gordon, 1986). Intrathecal administration of ATP produces long-lasting mechanical allodynia due to activation of $P2X_{2/3}$ receptors (Nakagawa et al., 2007). $P2X_3$ receptors are upregulated in DRG and spinal cord following nerve injury in regions corresponding to the inputs of the injured nerve, and administration of $P2X_3$ agonists following nerve injury increases the thermal hyperalgesia and allodynia that develops (Chen et al., 2001; Novakovic et al., 1999; Zhou et al., 2001). Knockdown of $P2X_3$ using spinally administered antisense oligonucleotides reduces αβ-MeATP-induced hyperalgesia, inflammatory hyperalgesia, and nerve injury-induced hyperalgesia (Baumann et al., 2004; Dorn et al., 2004; Honore et al., 2002). Systemically-administered $P2X_3$ antagonist reduces inflammatory pain and nerve-injury induced allodynia (Jarvis et al., 2002a). While all of these studies suggest a strong pro-nociceptive role for $P2X_3$ receptors, complicating matters is the fact that $P2X_3$ null mice show normal acute thermal and mechanical sensitivity, have normal responses to carrageenan and capsaicin, but develop greater thermal hyperalgesia due to CFA (Souslova et al., 2000). The reasons for discrepancies between studies using acute inhibition of the receptor by

antagonists or antisense oligonucleotide knockdown and those in $P2X_3$ null mice could be due to developmental compensation in the knockout mice.

Recently, an important role for $P2X_4$ receptors has been shown in the development of neuropathic pain following nerve injury (Tsuda et al., 2003). $P2X_4$ receptors are expressed in both neuronal and non-neuronal tissues, but are strongly upregulated in microglia following peripheral nerve injury (Khakh et al., 2001; Tsuda et al., 2003). Intrathecal administration of ATP-activated microglia produced allodynia through $P2X_4$ receptors (Tsuda et al., 2003). These actions require signaling through MAPK and subsequent release of cytokines and chemokines that sensitize neighboring neurons (Tsuda et al., 2005; Tsuda et al., 2004). Administration of $P2X_4$ antagonists or knockdown of the receptor using antisense oligonucleotides reduces nerve-injury induced neuropathic pain (Tsuda et al., 2003). Interestingly, chronic inflammation does not lead to $P2X_4$ upregulation or microglial activation (Rabchevsky et al., 1999). $P2X_4$ knockout mice show normal acute nocifensive responses in several assays, but develop significantly less mechanical allodynia following inflammation by CFA or nerve injury (Tsuda et al., 2009). These studies show $P2X_4$ receptors to be important targets for the treatment of chronic pain.

Microglia and macrophages express $P2X_7$ receptors. Activation of these receptors leads to release of inflammatory cytokines, particularly interleukin-1β (IL-1β), which can lead to upregulation of other pro-nociceptive compounds, such as NGF and Cox-2 (North, 2002; Wirkner et al., 2007). Systemic administration of $P2X_7$ antagonists significantly reduces nociception in models of inflammatory and neuropathic pain, predominantly through blockade of IL-1β release (Honore et al., 2006; McGaraughty et al., 2007; Nelson et al., 2006). While $P2X_7$ knockout mice have normal acute nociception, they fail to develop

chronic inflammatory or neuropathic pain states following injection of CFA or nerve injury, respectively (Chessell et al., 2005). This, again, is due to decreased release of IL-1β and other inflammatory mediators. Since $P2X_7$ seems to play a key upstream role in a transduction pathway common to several pain states, it is seen as a very promising target for the treatment of chronic pain.

1.5.1.2) P2Y receptors

Unlike P2X receptors, there have been few studies examining the role of P2Y receptors in modulation of nociception. Even the specific P2Y receptors expressed in DRG is controversial, as early studies showed mRNA expression of $P2Y_2$ and $P2Y_4$ (Okada et al., 2002), but more recent studies suggest expression of $P2Y_1$ and $P2Y_2$, but not $P2Y_4$ mRNA in DRG neurons (Kobayashi et al., 2006). Non-neuronal DRG cells express $P2Y_2$, $P2Y_{12}$, and $P2Y_{14}$ receptors (Wirkner et al., 2007). P2Y receptors can be studied using the agonists UTP and UDP, which avoid concomitant activation of P2X receptors. UTP evokes sustained action potential firing in some C fibers, most likely through $P2Y_1$ and $P2Y_2$ activation (Okada et al., 2002; Stucky et al., 2004). Further, $P2Y_1$ and $P2Y_2$ activation is thought to sensitize TRPV1 through phosphorylation (Moriyama et al., 2003; Tominaga et al., 2001). However, activation of excitatory $P2X_3$ and N-type calcium channels is inhibited by the $P2Y_1$ agonist ADP (Gerevich et al., 2005; Gerevich et al., 2007). In addition, intrathecal administration of UTP and UDP has antinociceptive effects in both thermal and mechanical assays (Okada et al., 2002). UTP and UDP injection also produces antiallodynic effects after sciatic nerve ligation. Thus, while activation of $P2Y_1$ and $P2Y_2$ receptors may provoke neuronal firing in vitro, it appears to have an antinociceptive effect in vivo.

Recently, $P2Y_{12}$ receptors on microglia were shown to be necessary for development of neuropathic pain following nerve injury (Haynes et al., 2006). In addition, intrathecal delivery of a $P2Y_{12}$ antagonist prevents development of tactile allodynia following nerve injury (Tozaki-Saitoh et al., 2008). The pro-nociceptive effects of $P2Y_{12}$ activation most likely occurs through activation of the p38 MAPK pathway, similar to $P2X_4$ (Kobayashi et al., 2008). Based on these data, development of selective $P2Y_{12}$ antagonists could prove helpful in treatment of neuropathic pain following nerve injury.

1.5.2) Adenosine in Nociception

Adenosine regulates nociceptive neurotransmission at spinal, supraspinal, and peripheral sites (Dickenson et al., 2000; Sawynok and Liu, 2003). This regulation is important in the initiation and maintenance of inflammatory and neuropathic pain. Adenosine exerts its effects through activation of the four subtypes of P1 receptors – A_1, A_{2A}, A_{2B}, and A_3 (Abbracchio et al., 2009; Burnstock, 2009). These are seven-transmembrane domain GPCRs that couple to different types of G proteins. A_1 and A_3 receptors couple to G_i or G_o proteins, which inhibit cAMP production and activate or enhance phospholipase C activity (Jacobson and Gao, 2006; Murthy and Makhlouf, 1995b). A_{2A} and A_{2B} receptors couple to G_s (and occasionally G_q), and their activation stimulates production of cAMP. All four classes of receptors show a widespread distribution in the nervous system and play a role in nociception. However, not all effects of adenosine are due to receptor activation at neuronal sites. Direct modulation of signaling through nociceptors and transmission neurons occurs predominantly through activation of A_1 and A_{2A} receptors, while indirect effects on

pain transmission occur through activation of A_{2A}, A_{2B}, and A_3 receptors on glia in the CNS and inflammatory cells at peripheral sites (Sawynok and Liu, 2003).

1.5.2.1) A_1 Adenosine Receptors

Delivery of adenosine analogs via supraspinal, spinal, and systemic routes leads to antinociceptive effects, due largely to activation of A_1Rs. A_1Rs are expressed on DRG and trigeminal ganglion neurons (predominantly small- and medium-diameter), as well as at postsynaptic sites in the dorsal horn of the spinal cord, in lamina II_{inner} near $IB4^+$ terminals (Carruthers et al., 2001; Schulte et al., 2003). Activation of A_1Rs in vitro results in reduced Ca^{2+} entry, decreased cAMP generation, and decreased substance P and CGRP release from DRG neurons (Carruthers et al., 2001; Haas and Selbach, 2000; Santicioli et al., 1993; Sjolund et al., 1997). In addition, activation of A_1Rs in the substantia gelatinosa of the dorsal horn of the spinal cord inhibits neurotransmission through both pre- and postsynaptic mechanisms (Lao et al., 2001; Li and Perl, 1994; Patel et al., 2001). The postsynaptic mechanism is due to an increase in K^+ channel activity, leading to increased hyperpolarization and decreased action potential firing in secondary spinal cord neurons (Li and Perl, 1994; Salter et al., 1993). This mechanism leads to inhibition of substance P and excitatory amino acid-evoked activity, and is important in the inhibition of wind-up seen following application of A_1R agonists (DeLander and Wahl, 1991; Doi et al., 1987; Reeve and Dickenson, 1995). In addition, A_1R activation can augment the antinociceptive actions of morphine under some conditions, and blockade of A_1Rs can inhibit morphine antinociception at the spinal level (Contreras et al., 1990; DeLander and Hopkins, 1986; Jurna, 1984; Malec and Michalska, 1988).

Activation of A_1R also leads to antinociceptive effects in the periphery in animals. Local administration of A_1R agonists into the hindpaw of rats leads to antinociception in a pressure hyperalgesia model, the formalin model, inflammatory models, and following nerve injury (Aley et al., 1995; Aumeerally et al., 2004; Karlsten et al., 1992; Liu and Sawynok, 2000; Taiwo and Levine, 1990). Increases in peripheral adenosine due to blockade of adenosine deaminase also lead to antinociception through A_1R activation (Liu and Sawynok, 2000; Sawynok, 1998). These antinociceptive effects are due to inhibition of adenylate cyclase and subsequent decreases in intracellular cAMP (Carruthers et al., 2001; Taiwo and Levine, 1990). Importantly, the antinociceptive properties of peripheral A_1R activation can be counteracted by activation of pro-nociceptive A_{2A}, A_{2B}, and A_3 receptors (see below).

In humans, a more complex situation is present. Application of adenosine to human blister base, as well as i.v. administration of adenosine by bolus injection causes pain (Bleehen and Keele, 1977; Crea et al., 1992; Crea et al., 1990; Sylven et al., 1988; Sylven et al., 1986). Some studies suggest these effects are due to activation of A_1R and subsequent increase in C-fiber activity (Dowd et al., 1998a; Gaspardone et al., 1995; Hong et al., 1998; Kirkup et al., 1998; Pappagallo et al., 1993). These findings are at odds with those showing peripheral antinociception through A_1R, and further studies are needed to determine the role of A_1Rs in human nociception.

Centrally, spinal application of A_1R agonists is antinociceptive in models of acute, inflammatory, and neuropathic pain (for review, see Dickenson et al., 2000; Sawynok, 1998). These actions can be blocked by A_1R antagonists (Gomes et al., 1999; Lee and Yaksh, 1996; Poon and Sawynok, 1998), and are due to effects of A_1R activation on postsynaptic K^+ channel activity and inhibition of pre-synaptic glutamate and neuropeptide release

(Carruthers et al., 2001; Lao et al., 2001; Li and Perl, 1994; Patel et al., 2001; Sjolund et al., 1997). In addition to direct activation by agonists, A_1R can be activated by increased endogenous adenosine levels through use of inhibitors of adenosine metabolism, namely adenosine kinase and adenosine deaminase inhibitors. These drugs are also antinociceptive in models of inflammatory and neuropathic pain states, due to A_1R activation (Jarvis et al., 2002c; Kowaluk and Jarvis, 2000; McGaraughty et al., 2001; McGaraughty et al., 2005). Direct administration of adenosine itself has long-lasting analgesic properties when delivered centrally in neuropathic rats and humans (Belfrage et al., 1999; Lavand'homme and Eisenach, 1999). Spinal administration of adenosine produces analgesia that is specific for sensitized states, while intravenous adenosine reduces experimental, post-operative, and neuropathic pain in humans (Belfrage et al., 1999; Eisenach et al., 2002; Segerdahl et al., 1995a; Segerdahl et al., 1995b; Segerdahl et al., 1994; Segerdahl et al., 1996; Sollevi et al., 1995). All of these data suggest great potential for targeting of A_1R for treatment of chronic pain. However, studies using high doses of A_1R-selective agonists have shown side effects, including motor paralysis and autonomic dysfunction (Sawynok, 1998). The motor side effects are likely due to activity at A_1 or A_2 receptors on motor neurons and could be reduced or eliminated by usage of lower doses or modulators of endogenous adenosine metabolism (Reppert et al., 1991).

Knockout mice for A_1R ($A_1R^{-/-}$) further show the important role of this receptor in nociception. These mice show enhanced thermal sensitivity in the tail flick assay, but have normal responses to mechanical or cold stimuli at baseline (Johansson et al., 2001; Wu et al., 2005). They also show increased thermal hyperalgesia (both hot and cold) following inflammation (carrageenan) or nerve injury (ischemic sciatic nerve injury), but normal

mechanical allodynia (Wu et al., 2005). $A_1R^{-/-}$ mice also have reduced antinociception in response to intrathecal R-PIA (an A_1R agonist) and morphine (Wu et al., 2005). Taken together these data confirm an important role for A_1R in the modulation of thermal nociception and morphine analgesia.

1.5.2.2) A_{2A} Adenosine Receptors

A_{2A} receptors are expressed in DRG neurons and glial cells, but are traditionally thought to be absent from the spinal cord (Cunha et al., 2006; Hasko et al., 2005; Hussey et al., 2007; Kaelin-Lang et al., 1998). However, a recent study suggests the possible expression of the receptor in spinal cord, where it may affect nociception (Bura et al., 2008). Signaling through these receptors is predominantly pro-nociceptive. Local peripheral application of $A_{2A}R$ agonists increases mechanical hyperalgesia and flinching in response to formalin, and application of $A_{2A}R$ antagonists reduces nociceptive behavior (Doak and Sawynok, 1995; Khasar et al., 1995; Taiwo and Levine, 1990). The pro-nociceptive effects following receptor activation are due to increased cAMP levels, leading to activation of PKA and modulation of Na^+ channels (Gold et al., 1996; Khasar et al., 1995; Taiwo and Levine, 1991). The role of $A_{2A}Rs$ centrally is unclear, as spinal application of both $A_{2A}R$ agonists and antagonists produce antinociception (Godfrey et al., 2006; Hussey et al., 2007; Ledent et al., 1997; Regaya et al., 2004; Yoon et al., 2005). $A_{2A}R$ knockout mice ($A_{2A}R^{-/-}$) show reduced thermal nociceptive responses in a number of tests, reduced nocifensive responses to formalin (both phases), reduced thermal hyperalgesia and mechanical allodynia following nerve injury, as well as changes in responses to opioids (Bailey et al., 2002; Berrendero et al., 2003; Bura et al., 2008; Godfrey et al., 2006; Ledent et al., 1997). These results support a

pro-nociceptive role for A_{2A}Rs. The effects following nerve injury corresponded with decreased activation of microglia and astrocytes in $A_{2A}R^{-/-}$ mice, suggesting a possible role of A_{2A}Rs on glial cells in modulation of nerve injury-induced pain (Bura et al., 2008).

1.5.2.3) A_{2B} and A_3 Adenosine Receptors

A_{2B} and A_3 receptors are expressed peripherally on mast cells, and their activation has pro-nociceptive effects. This is predominantly due to subsequent release of mast cell mediators, such as histamine and serotonin (Sawynok et al., 1997). Local application of A_3R agonists leads to nociceptive responses similar to those seen following formalin injection, while systemic A_3R agonists produce thermal hyperalgesia (Abo-Salem et al., 2004; Sawynok et al., 1997). Systemic application of an A_{2B}R antagonist reduces thermal nociception due to peripheral receptor blockade (Abo-Salem et al., 2004). Surprisingly, A_{2B}R knockout mice have not yet been tested for changes in nociception. A_3R knockouts show normal thermal and mechanical thresholds at baseline, although there is one report of reduced thermal nociception in the hot plate test (Fedorova et al., 2003; Wu et al., 2002). These mice show reduced heat hyperalgesia, plasma extravasation, and edema following injection of carrageenan, suggesting a pro-inflammatory role for A_3R (Wu et al., 2002). Further study of these receptors is needed to fully understand their activity in nociceptive signaling.

1.5.3) Sources of Endogenous Adenosine

As a result of the importance of adenosine and adenine nucleotides in the modulation of neuronal function, there are a number of mechanisms involved in the production, release,

and regulation of these molecules in the nervous system. Surprisingly, there is a substantial amount of extracellular adenosine present in many systems, enough to activate high affinity adenosine receptors (A_1 and A_{2A}) at basal levels (Dunwiddie and Masino, 2001). This activation leads to the "purinergic tone" seen in these systems. This tone explains the effects of drugs that block these receptors at baseline, such as caffeine and methylxanthines. In the spinal cord, it partially explains why mice null for A_1R show a nociceptive phenotype at baseline (Johansson et al., 2001; Wu et al., 2005). Extracellular adenosine primarily arises from two general sources (Figure 1.5): 1) release of adenosine from the intracellular space via several mechanisms and 2) dephosphorylation of extracellular adenine nucleotides (ATP, ADP, and AMP).

Adenosine can be produced intracelluarly from several sources and released via multiple mechanisms. Intracellular concentrations of ATP are approximately 50 times higher than those of AMP, creating a gradient for the breakdown of ATP by ATPases to AMP, which is then acted upon by intracellular 5'-nucleotidase ((cN)-I) to produce adenosine (Latini and Pedata, 2001; Sawynok and Liu, 2003). In addition, adenosine can be made by conversion of intracellular cyclic AMP (cAMP). This cAMP is made following activation of cellular GPCRs, and is first converted to AMP by phosphodiesterase, then broken down to adenosine by (cN)-I (Brundege et al., 1997; Rosenberg and Li, 1995). Finally, adenosine can be made from S-adenosyl-homocysteine (SAH) by SAH hydrolase (Latini and Pedata, 2001). The role of this pathway in the spinal cord is not known. As stated above, this intracellular adenosine can be directly released into the intracellular environment in response to several stimuli, including elevated K^+, veratridine (opens Na+ channels, modeling neuronal activation), substance P, glutamate, and morphine (Cahill et al., 1993; Cahill et al., 1997;

Conway et al., 1997; Sandner-Kiesling et al., 2001; Sweeney et al., 1987a; Sweeney et al., 1987b). In addition, under conditions of high intracellular adenosine, it can pass into the extracellular space through equilibrative nucleoside transporters. The properties and mechanisms of release in response to these stimuli and conditions are reviewed extensively in Sawynok and Liu (2003).

There are multiple mechanisms whereby adenine-containing nucleotides can be released into the extracellular space of the spinal cord (for review, see Yegutkin, 2008). ATP is a well-known neurotransmitter, and can be co-localized with serotonin, acetylcholine, dopamine, or norepinephrine (Dunwiddie and Masino, 2001). Upon neuronal stimulation, ATP is released and can act via its receptors (see above). In the spinal cord, ATP is released from sensory afferent terminals via spinal cord synaptosomes (Sawynok et al., 1993; Sawynok and Liu, 2003). It is also co-released with γ-aminobutyric acid (GABA) from spinal cord interneurons, and from glial cells following activation of ionotropic glutamate receptors (Jo and Schlichter, 1999; Queiroz et al., 1997). Once in the extracellular space, ATP is quickly dephosphorylated to terminate its action at P2X and P2Y receptors. This process can involve several enzymes, including ecto-nucleoside triphosphate diphosphorylases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), Ecto-5'-nucleotidase (NT5E), and alkaline phosphatases (ALP) (Yegutkin, 2008; Zimmermann, 2006). The final product of this process is adenosine, which can act on adenosine receptors, be converted extracellularly to inosine, or be taken back up into cells via equilibrative nucleoside transporters. Once inside the cell, adenosine is phosphorylated to AMP by adenosine kinase (AK) or deaminated to inosine by adenosine deaminase (AD). Blockade of AK or AD increases basal and evoked adenosine release in vivo and in vitro,

and leads to antinociceptive effects through activation of A_1R (Latini and Pedata, 2001). The extracellular production of adenosine by this nucleotide degradation pathway is extremely pertinent to my work and is discussed in greater detail below.

1.5.4) Ectonucleotidases in Nociception

As discussed above, the extracellular production of adenosine relies on the activity of several different enzymes that can degrade adenine-containing nucleotides in a step-wise manner. These enzymes thus play important roles in the regulation of both P1 (through production of adenosine) and P2 (through degradation of ATP and ADP) receptor activation. Since their activity can decrease activation of pro-nociceptive receptors (P2X) and increase activation of antinociceptive receptors (A_1R), these enzymes may play an important role in the modulation of nociceptive circuits. Surprisingly, the molecular identity and function of specific ectonucleotidases in the spinal cord and DRG was not significantly studied prior to this dissertation work. The identification and characterization of these ectonucleotidases could provide novel therapeutic targets for the treatment of acute, inflammatory, or neuropathic pain. The ectonucleotidases that metabolize nucleotides in other regions of the nervous system are highlighted below, and are further reviewed by Zimmermann (2006), as well as Yegutkin (2008).

1.5.4.1) Ecto-nucleoside triphosphate diphosphorylases (E-NTDPases)

Eight different E-NTPDase genes have been identified in mammals (NTPDase1-8), four of which are expressed on the cell surface – NTPDase1, 2, 3, and 8 (Zimmermann, 2006). NTPDase5 and 6 are secreted, while NTPDase4 and 7 are intracellular and face the

lumen of cytoplasmic organelles (Yegutkin, 2008). The cell-surface family members (1, 2, 3, and 8) are highly glycosylated and contain two predicted transmembrane domains at the N- and C-termini. They also contain five highly-conserved sequence domains, known as "apyrase conserved regions" (ACR) (Robson et al., 2006; Smith and Kirley, 1999). They may exist on the cell surface as monomers or homooligomers (dimeric, trimeric, or tetrameric) (Stout and Kirley, 1996).

The NTPDases can hydrolyze a variety of nucleoside di- and triphosphates, but preferences and rates of reaction differ between the enzymes. These properties are of considerable significance for the regulation of nucleotide signaling. All four surface-expressed NTPDases can degrade ATP and UTP, but have varying degrees of activity towards nucleoside diphosphates (Zimmermann, 2006). Indeed, the ATP:ADP hydrolysis ratios for NTPDase1, 2, 3, and 8 vary widely: ~1.3; ~20-30; ~4; ~1.6, respectively (Kukulski et al., 2005; Zimmermann, 2001b). However, recent studies suggest that the estimated ATPase activity of these enzymes is overestimated by traditional methods, and more reliable tests show much higher ADPase activity (Marcus et al., 1997; Yegutkin et al., 2001). Regardless, NTPDase2 and 3 show much higher preference for triphosphates than diphosphates. The differences in catalytic properties are better understood by examining product formation, as well as substrate degradation. NTPDase1 directly converts ATP to AMP without significant ADP production, bypassing potential nucleoside diphosphate receptor (P2Y) activation (Zimmermann, 2006). However, UTP hydrolysis by NTPDase1 does lead to significant UDP production (Kukulski et al., 2005). Unlike NTPDase1, NTPDase2 makes ADP from ATP, which is then slowly converted to AMP, allowing for more extensive diphosphate receptor activity. NTPDase3 and 8 have intermediate patterns of

product formation (Kukulski et al., 2005; Zimmermann, 2001b). These catalytic preferences and activities are important in the degradation of P2X and production of P2Y receptor substrates.

All four surface-located E-NTPDases show overlapping tissue distribution, but none are expressed in the same cells (Bigonnesse et al., 2004; Zimmermann, 2001a). In the nervous system, NTPDase1 is expressed at the surface of endothelial and smooth muscle cells and microglia (Braun and Zimmermann, 2001). NTPDase2 is in neural progenitors of the brain, immature and non-myelinating Schwann cells, and satellite glia in DRG and sympathetic ganglia (Braun et al., 2003; Braun et al., 2004; Shukla et al., 2005). NTPDase3 is in the brain, but specific cellular localization is unknown, while expression of NTPDase8 is either low or absent in the brain (Bigonnesse et al., 2004; Lavoie et al., 2004; Smith and Kirley, 1999; Vorhoff et al., 2005). A recent attempt to localize these enzymes by in situ hybridization in the DRG and spinal cord in our lab has revealed that NTPDase3 is highly expressed in DRG neurons of all sizes, as well as in motor neurons of the ventral horn of the spinal cord. There is weak expression in laminae I and II of the dorsal horn. Expression of NTPDase1, 2, or 8 in these regions is either low or absent. Further studies are needed to determine the importance of these enzymes in nociception.

1.5.4.2) Ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs)

There are seven related mammalian E-NPPs (NPP1-7), but only three – NPP1, NPP2, and NPP3 – can degrade various nucleotides (Yegutkin, 2008). NPP1 and NPP3 are type II transmembrane glycoproteins, while NPP2 is produced as a pre-pro-enzyme and is secreted (Robson et al., 2006; Yegutkin, 2008). Physiological substrates of these enzymes include

NAD, nucleotide sugars, ATP, and dinucleoside polyphosphates (Bollen et al., 2000; Goding et al., 2003; Vollmayer et al., 2003; Zimmermann, 2000). Hydrolytic activity of these enzymes reaches optimum at very alkaline pH, similar to that of alkaline phosphatase. All three enzymes can degrade both nucleoside tri- and diphosphates. Hydrolysis of ATP proceeds directly to AMP, bypassing production of ADP and nucleoside diphosphate receptor activation (Zimmermann, 2006). In addition, all three enzymes also degrade diadenosine polyphosphates (Ap_3A, Ap_4A, and Ap_5A) at comparable rates and are the most important enzymes in metabolism of extracellular diadenosine polyphosphates (Vollmayer et al., 2003). Hydrolysis of these substrates is asymmetric, producing AMP and Ap_{n-1}, meaning this activity could also produce adenosine tetraphosphate, ATP, or ADP, and cause subsequent P2 receptor activation (Rotllan et al., 2002). Thus, similar to E-NTPDases, E-NPPs can either hydrolyze or produce ligands for nucleotide receptors, potentially modulating nociceptive signaling. In the nervous system, NPP1 is found in capillaries of the brain, NPP2 is in choroid plexus, and NPP3 is associated with a specific subset of brain glial precursors (Goding et al., 2003; Zimmermann, 2006). Expression in DRG or spinal cord has not been studied, and their physiological relevance in nociceptive signaling is unknown.

1.5.4.3) Alkaline Phosphatases (APs)

Of the four known alkaline phosphatases (intestinal AP, placental AP, germ cell AP, and tissue non-specific AP (TNAP)), only TNAP is thought to be expressed in neuronal tissues (Zimmermann, 2006). APs are glycosylphosphatidyl inositol (GPI)-anchored proteins and act as non-specific phosphomonoesterases. They can act on numerous phosphorylated compounds, including adenine nucleotides, pyrophosphate, phosphatidates with various fatty

acid chains, inorganic polyphosphates, β-glycerophosphate, and glucose-phosphates (Fonta et al., 2005; Narisawa et al., 1994; Yegutkin, 2008). As the name would suggest, activity of the enzymes is greatest at alkaline pH (8 – 11). Since TNAP can perform the entire stepwise hydrolysis of nucleoside triphosphates to nucleoside (i.e., ATP → ADP → AMP → Adenosine), it could have a profound role on the regulation of both P1 and P2 receptors. TNAP is expressed in the neural tube and in various brain regions during late-stage embryonic development, and in neuropil of various regions in adult mice (Fonta et al., 2005; Narisawa et al., 1994). Recent studies in our lab suggest TNAP is expressed in all regions of the gray matter of the spinal cord. However, the role of TNAP in nervous system function has not been studied in detail.

1.5.4.4) Ecto-5'-nucleotidase (NT5E)

In humans, there are seven known 5'-nucleotidases, but only one version (NT5E, also known as CD73) is expressed on the cell surface as a GPI-anchored enzyme (Yegutkin, 2008). This enzyme is made of two 60-70 kDa glycoprotein subunits tethered together by non-covalent bonds, which are necessary for enzyme activity (Martinez-Martinez et al., 2000). The enzyme binds to divalent metal cations which can have stimulatory (Zn^{2+} or Mg^{2+}) or inhibitory (Pb^{2+} and Hg^{2+}) effects on activity (Fini et al., 1990; Ong et al., 1990). NT5E selectively hydrolyzes nucleoside 5'-monophosphates, and has little or no activity towards nucleoside 2'- or 3'-monophosphates (Hunsucker et al., 2005; Zimmermann, 1992). The enzyme has a K_m towards AMP and IMP in the low micromolar range (1 – 50 μM), and V_{max}/K_m ratios show 5'-AMP to be the best substrate for the enzyme (Zimmermann, 1992). NT5E can be inhibited competitively by ATP, ADP, and adenosine 5'-[α,β-

methylene]diphosphate and non-competitively by concanavalin A (Zimmermann, 1992). In addition, some reports suggest that methylxanthines like theophylline and caffeine also inhibit NT5E (Fredholm et al., 1978; Heyliger et al., 1981). The pH optimum for enzyme activity is in the range of 7 – 8.

While some studies suggest a role of NT5E in intracellular signaling and mediation of cell-cell and cell-matrix adhesions (Airas et al., 1997; Resta et al., 1998), the major physiological role of the enzyme is the regulation of purinergic signaling. Indeed, NT5E is likely to be the most important, rate-limiting step in the conversion of extracellular ATP to adenosine in many systems (Dunwiddie et al., 1997). This activity leads to decreased stimulation of P2 receptors and increased stimulation of P1 receptors. Through this function, NT5E is important in a number of physiological functions, including epithelial ion and fluid transport, maintenance of tissue barriers (i.e., intestinal or vascular permeability), ischemic preconditioning in the heart and kidneys, adaptation to hypoxia, and anti-inflammatory effects on neutrophils (for review, see Colgan et al., 2006). Indeed, studies using mice null for NT5E (*Nt5e*$^{-/-}$) have confirmed roles for the enzyme in the mediation of tubuloglomerular feedback and renal function (Castrop et al., 2004; Huang et al., 2006a), cardioprotection during myocardial ischemia (Eckle et al., 2007c), immunomodulatory and thromboregulatory responses (Koszalka et al., 2004; Thompson et al., 2004a; Zernecke et al., 2006), pulmonary integrity and lung function (Eckle et al., 2007a; Volmer et al., 2006), and maintenance of vascular barrier function during hypoxia (Thompson et al., 2004a). Surprisingly, while purinergic signaling is extremely important for nervous system function, NT5E has not been studied in this context.

NT5E is thought to be expressed in a number of different tissues, including liver, heart, blood vessels, lung, colon, kidney, brain, and spinal cord (Moriwaki et al., 1999; Yegutkin, 2008; Zimmermann, 1992; Zimmermann, 1996). Expression has been largely based on biochemical or histochemical assays, as antibodies against the protein have either been inadequate for tissue localization or shown conflicting results. For example, in the brain, enzyme histochemistry suggests broad NT5E expression in many locations in developing synapses, sprouting nerve fibers, and microglia, while immunocytochemical studies suggest expression only in glia, choroid plexus, and vascular epithelium (Zimmermann, 1996). A recent study using enzyme histochemical assays in *Nt5e*$^{-/-}$ mice confirmed broad expression of the enzyme in the brain, including the hippocampus, the cingulate cortex, the caudoputamen, the olfactory bulb and cortex, and the cerebellum (Langer et al., 2008). Despite this, a functional role for NT5E in any of these brain regions has not been shown.

A few studies suggested NT5E may be expressed and functional in the spinal cord and DRG. Early enzyme histochemical studies showed strong NT5E-like activity in the substantia gelatinosa along the total length of the spinal cord in mice and rats, predominantly in laminae II and III (Suran, 1974). In DRG, these studies showed NT5E-like activity in most neurons, although staining in large-diameter neurons was different in quality from staining in small-diameter neurons (Nagy and Daddona, 1985). In addition, more recent studies examined a possible role for NT5E activity in the modulation of nociception. Two studies suggested that NT5E-like activity was decreased in spinal cord synaptosomes following repeated stress, and this change led to hyperalgesia to thermal stimuli (Fontella et al., 2005; Torres et al., 2002). One study showed hypothyroidism-induced reduction in

thermal nociception corresponds with decreased NT5E-like activity in the spinal cord (Bruno et al., 2005). An additional study used a push-pull microprobe to study NT5E-like activity in the spinal cord in vivo, and showed that adenosine production was likely to be partially due to NT5E activity, based on the use of a selective inhibitor of the enzyme (Patterson et al., 2001). This NT5E-like activity reversed capsaicin-induced nociception (Patterson et al., 2001). While these anatomical and functional studies suggest NT5E is present and active in nociceptive circuits, a major caveat overshadows this work. All of these studies used degradation of the substrate 5'AMP (or another 5'-monophosphate) to measure the presence or activity of NT5E. While NT5E can certainly perform this reaction, it is not the only enzyme capable of doing so. Indeed, AP (see above) and FRAP (see below) can both dephosphorylate 5'AMP to adenosine and are thought to be expressed in DRG and spinal cord. Thus, it cannot be excluded that the observed AMPase activity in any of these studies was due to another surface enzyme besides NT5E. Further studies using *Nt5e*$^{-/-}$ mice or more specific NT5E inhibitors may help clarify the enzyme's role in nociception.

1.5.4.5) Fluoride resistant acid phosphatase (FRAP)

Fifty years ago, Colmant recognized that small- and medium-diameter sensory neurons could be labeled using a technique that examined the degradation of a phosphorylated substrate by an acid phosphatase (1959). This acid phosphatase was subsequently shown to be relatively resistant to inhibition by fluoride (unlike a known lysosome-located acid phosphatase), and was named "fluoride-resistant acid phosphatase" or FRAP. FRAP was localized specifically to C- and some Aδ-fiber nociceptors in the DRG, and to axonal terminals in lamina II of the dorsal spinal cord (Csillik and Knyihar-Csillik,

1986). Later studies revealed that FRAP could also degrade a number of substrates, including many 5'-nucleoside monophosphates (UMP, GMP, IMP, AMP, and TMP) (Dodd et al., 1983; Dodd and Jessell, 1982; Silverman and Kruger, 1988a). In fact, the ability to specifically degrade 5'thiamine monophosphate (TMP) led the enzyme to also be called "TMPase" (Knyihar-Csillik et al., 1986).

FRAP was used by many labs as a marker of nociceptive neurons. FRAP staining is seen specifically in the cell bodies of small- to medium-diameter DRG neurons, in central synaptic terminals of the substantia gelatinosa of the spinal cord at all levels, as well as in Lissauer's tract in the spinal cord, in the trigeminal ganglion in the brainstem, and in peripheral somatic and autonomic nerves (Coimbra et al., 1974; Csillik and Knyihar-Csillik, 1986; Dalsgaard et al., 1984). Examination of colocalization with neuropeptides and other markers showed FRAP was a selective marker for nonpeptidergic nociceptors in several mammalian species, including mice, rats, rabbits, dogs, cats, cows, monkeys, and humans (Dodd et al., 1983; Nagy and Hunt, 1982; Ribeiro-Da-Silva et al., 1986; Silverman and Kruger, 1988a). While FRAP activity is most intense at acidic pH (~5), it is also active at neutral pH (~7) (Silverman and Kruger, 1988a). Importantly, FRAP activity is membrane-localized in the plasma membrane, golgi, and endoplasmic reticulum in DRG and axon terminals of the substantia gelatinosa, but not in lysosomes where lysosomal acid phosphatase is found (Csillik and Knyihar-Csillik, 1986; Knyihar-Csillik et al., 1986).

Interestingly, changes in FRAP activity are seen following anatomical or chemical changes in sensory circuits. After complete dorsal rhizotomy, a decrease in FRAP activity was seen in the dorsal spinal cord ipsilateral to the surgery, while activity in the contralateral cord was unchanged (Coimbra et al., 1974; Csillik and Knyihar-Csillik, 1986; Knyihar et al.,

1974). In fact, FRAP activity was completely eliminated at the spinal cord level corresponding to the injury site. Similarly, partial nerve injury or nerve crush led to decreased dorsal horn staining at sites corresponding to injury (Colmant, 1959; Csillik and Knyihar, 1978; Shields et al., 2003; Tenser, 1985; Tenser et al., 1991; Vadakkan et al., 2005). These studies suggest that the staining in the spinal cord is in terminals of dorsal root origin, and the loss of staining is due to the death of axon terminals in the spinal cord following nerve injury. Studies have used this disappearance of FRAP following injury as a technique to map projections of peripheral nerves to the spinal cord (Rustioni et al., 1972; Rustioni et al., 1971). Following ligature of the dorsal root, FRAP accumulation was seen at the ligature site closest to the DRG, suggesting the enzyme is made in the neuronal cell body and transported to spinal cord terminals (Csillik and Knyihar-Csillik, 1986). This is supported by studies showing decreased FRAP staining following interruption of axonal transport (Csillik and Knyihar-Csillik, 1982; Fitzgerald et al., 1984). Together, these studies suggest FRAP is made in a subset of DRG neurons and transported to the axons of these neurons.

Because it was found in nociceptive circuits, FRAP was intensively studied for a potential role in pain transmission. As mentioned above, nerve injury due to transection, ligation, or crush led to loss of FRAP activity. This loss of activity corresponded with the onset of neuropathic pain symptoms seen following nerve injury, suggesting decreased FRAP activity may contribute to the allodynia and hyperalgesia associated with neuropathic pain. In addition, neonatal administration of capsaicin led to a subsequent reduction in FRAP activity, suggesting a possible involvement in nociceptive neurotransmission (Fitzgerald, 1983; Nagy et al., 1981). Conversely, injection of the TRPA1-activating substance formalin increased FRAP activity ipsilateral to the injection (Kantner and Kirby, 1982). In addition,

FRAP activity was increased in spinal cords of rats in a model of adjuvant arthritis (Schoenen et al., 1985), as well as in a model of heat-induced cutaneous inflammation (Glykys et al., 2003), further suggesting a role for FRAP in modulation of inflammatory pain. These data, along with its localization led some to suggest a possible role for FRAP in metabolism of neurotransmitters involved in nociceptive signaling. In fact, its ability to act on specific nucleotides led some to suggest that FRAP might play a role in nucleotide or purinergic neurotransmission (Fyffe and Perl, 1984; Silverman and Kruger, 1988a; Stone, 1981). However, no studies examined the possibility of FRAP acting as an ectonucleotidase in nociceptive circuits.

Despite its promise as a potential player in nociception, research on FRAP waned in the 1990's for two primary reasons. First, the discovery of the ability of isolectin IB4 from *Griffonia simplicifolia* to also bind nonpeptidergic DRG neurons and colocalize extensively with FRAP provided a much simpler and more powerful method for identifying this subset of nociceptors (Silverman and Kruger, 1988b; Silverman and Kruger, 1990). Second, the gene for FRAP was never identified, making it impossible to perform immunohistochemical, molecular, and genetic studies that might further elucidate its role in nociception.

An attempt to identify the phosphatase responsible for FRAP activity was made by Dodd et al. who partially purified FRAP protein from rat DRG using column chromatography (1983). By binding the compound L-tartrate (a known inhibitor of FRAP) to a column, they were able to acquire a purified version of the functional enzyme. The electrophoretic mobility of the enzyme was identical to that of human prostatic acid phosphatase, (PAP) which also has similar substrate specificity to FRAP. They then made monoclonal antibodies against the purified FRAP protein, and tested these, as well as

antibodies against PAP in rat DRG and spinal cord. Unfortunately, none of the antibodies, including those directed against PAP, stained small-diameter DRG neurons or lamina II of the spinal cord where FRAP activity is located. This discrepancy prevented the authors from determining if PAP was indeed the enzyme responsible for FRAP activity. In addition, studies using neurotoxin treatment to target sensory neurons suggested that FRAP was of low molecular weight, resistant to tartrate, sensitive to fluoride inhibition, and associated with enzymes, all of which are inconsistent with PAP's characteristics (McDougal et al., 1985; McDougal et al., 1983). These findings cast further doubt on the possible relationship between FRAP and PAP. Despite these negative findings, recent work in our lab indicates that PAP isthe phosphatase responsible for FRAP activity in the DRG and spinal cord (see Chapter 2).

PAP is a member of the histidine acid phosphatase family of proteins, which contain a highly conserved R**H**GXRXP motif within their active sites (Van Etten, 1982). Mutation of the active site histidine residue (His12) to an alanine eliminates the phosphatase activity of the enzyme (Ostanin *et al.*, 1994). PAP is either expressed as a transmembrane (TM) protein with the active site on the extracellular side of the membrane, or as a secreted protein (Quintero et al., 2007). Both forms are homodimers with subunits related by a twofold axis (Schneider et al., 1993). The secreted form of the protein (S-PAP) is found mainly in the prostate and can be used as a marker for the detection of metastatic prostate cancer (Ostanin et al., 1994; Roiko *et al.*, 1990). The TM version of the protein has only been recently identified and is widely expressed in mouse tissues, including prostate, salivary gland, thymus, lung, kidney, brain, spleen, thyroid, and Schwann cells (Quintero et al., 2007).

When expressed heterologously, TM-PAP is localized to plasma membrane and intracellular vesicles.

As a result of its role in prostate cancer, the protein has been highly characterized. As its name suggests, PAP is optimally active at acidic pH, but functions over a broad pH range (~3 – 8) (Lam *et al.*, 1973; Van Etten, 1982). It is inhibited by L-tartrate, as well as a number of benzylaminophosphonic acids (Beers *et al.*, 1996; Van Etten, 1982). However, there are currently no known activators of the protein. PAP is a nonspecific phosphomonoesterase and can dephosphorylate a number of different substrates, including phosphoryl-o-tyrosine, phosphoryl-o-serine, β-glycerophosphate, para-nitrophenyl phosphate (p-NPP), lysophosphatidic acid, and many nucleotides, especially nucleotide monophosphates (i.e., TMP, AMP, GMP, CMP, UMP, XMP, and IMP) (Dziembor-Gryszkiewicz et al., 1978; Ostrowski and Kuciel, 1994; Silverman and Kruger, 1988a; Tanaka et al., 2004). The enzyme's K_m for AMP at pH 7 is in the low millimolar range. PAP has little to no activity on nucleoside di- or triphosphates at physiologic pH (Lam et al., 1973). The physiological roles of TM-PAP or S-PAP are unknown, although some studies suggest PAP may be involved in cell growth and division and that down-regulation of PAP may be involved in prostate cancer progression (Lin et al., 1994; Lin et al., 2001; Meng et al., 2000; Quintero et al., 2007).

1.6) Rationale for Dissertation Research

Adenosine and adenine-containing nucleotides clearly play important roles in the modulation of pain signaling. Despite this, knowledge of the enzymes responsible for the production and breakdown of these compounds in nociceptive circuits is minimal. Indeed,

despite hints from enzyme histochemistry, the molecular identity of any ectonucleotidases present in the DRG and spinal cord is unknown. This is surprising, given the importance these enzymes could have in decreasing signaling through P2X and P2Y receptors and in increasing signaling through P1 receptors. Knowledge of the identity of these proteins would allow for further manipulation and possible targeting for the development of novel therapeutic approaches for the treatment of acute or chronic pain.

The stepwise extracellular breakdown of ATP to adenosine can involve several possible enzymes and routes. Despite this, the clear rate-limiting step in this process is the breakdown of AMP into adenosine (Dunwiddie et al., 1997). Enhancing this reaction both increases the production of the anti-nociceptive compound adenosine and reduces the levels of the pro-nociceptive compound ATP. Thus, the enzymes responsible for this reaction in the spinal cord are of particular interest. It was the broad goal of this research project to molecularly identify and characterize the AMPases present in nociceptive circuits. Since some studies have already suggested potential expression and function of NT5E and FRAP/PAP in these regions, I will focus my studies on these enzymes. The experiments below are designed to address the following:

- Is PAP the molecular identity of FRAP?
- Are PAP and NT5E expressed in DRG neurons and spinal cord at the protein level?
- If PAP and NT5E are expressed in DRG, are they specific for particular classes of nociceptive neurons?
- Do PAP and NT5E act as ectonucleotidases in nociceptive circuits?
- Does PAP or NT5E activity lead to activation of adenosine receptors in nociceptive circuits?
- Does manipulation of endogenous PAP or NT5E affect nociception?

- Can soluble forms of PAP or NT5E be used to treat hyperalgesia or allodynia?

By addressing these questions, I intend to further the understanding of purinergic signaling in nociceptive circuits and to establish ectonucleotidases as viable targets for novel therapeutic approaches for the treatment of pain that are so desperately needed.

1.7) Figures and Tables

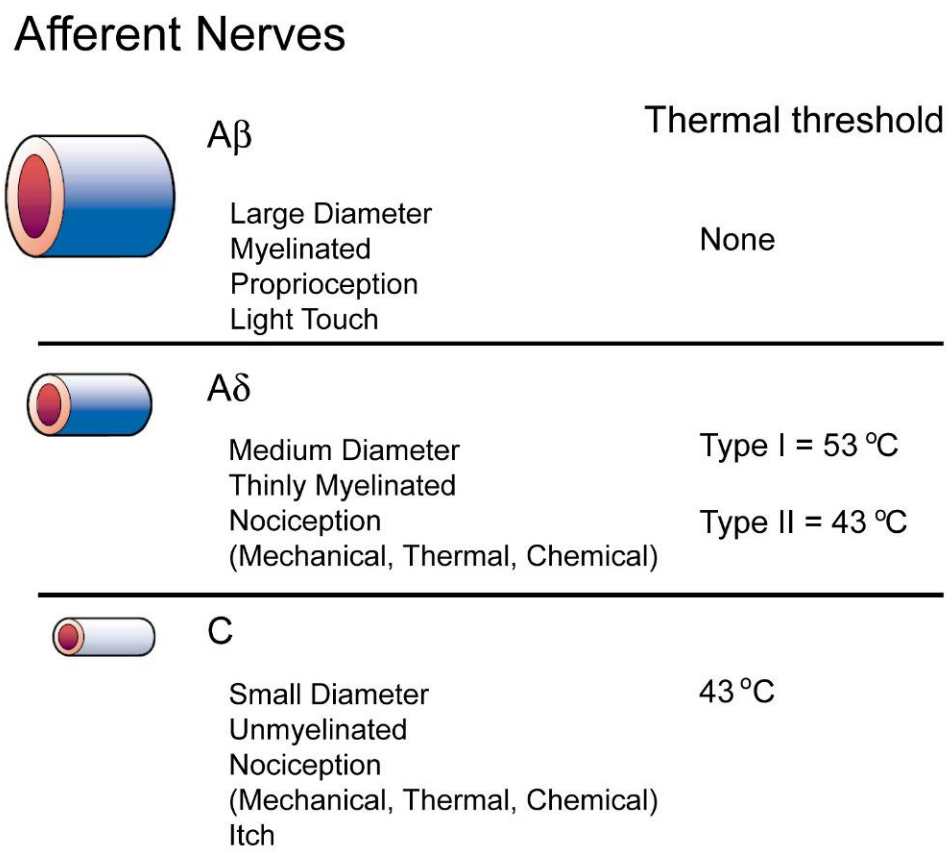

Figure 1.1. Primary afferent nerve types. Peripheral nerves have different properties and functions in detection of external stimuli. (Figure modified from Julius and Basbaum, 2001). (Back to text)

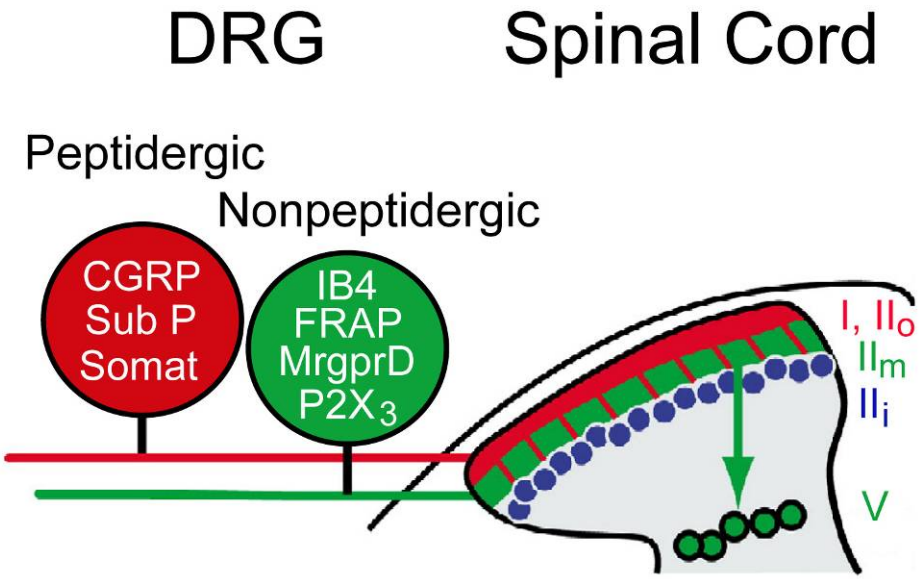

Figure 1.2. Nociceptive neurons and connections. Peptidergic neurons (red) express CGRP, substance P (Sub P), and Somatostatin (Somat) and project to laminae I and II_{outer} in the dorsal spinal cord. Nonpeptidergic neurons (green) stain for IB4 and FRAP activity, express *MrgprD* and $P2X_3$, and project to lamina II. Lamina $II_{m(iddle)}$ has been proposed as a subsection of lamina II_{inner}, where the majority of nonpeptidergic neurons appear to terminate (Zylka, 2005). (Figure modified from Zylka, 2005). (Back to text)

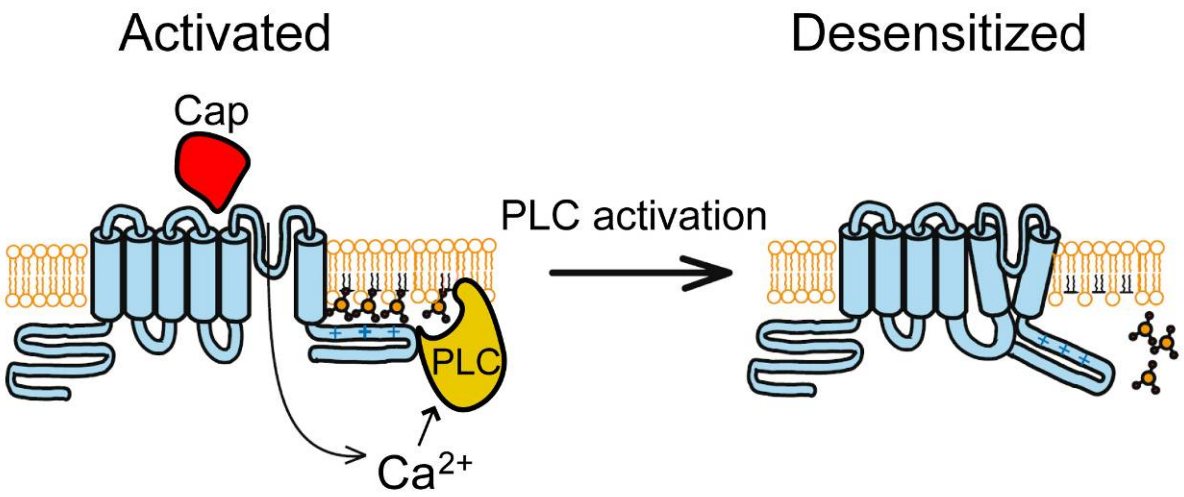

Figure 1.3. Desensitization of TRPV1 due to depletion of PIP_2. Activation of TRPV1 by Capsaicin (Cap) or heat causes influx of Ca^{2+} that activates phospholipase C (PLC). PLC degrades PIP_2 in the membrane into IP_3 and DAG, removing PIP_2 from its binding site on TRPV1. Loss of PIP_2 binding leads to inhibition of TRPV1 activity. This model does not take into account desensitization through calmodulin or calcineurin (see text). (Figure modified from Rohacs et al., 2008). (Back to text)

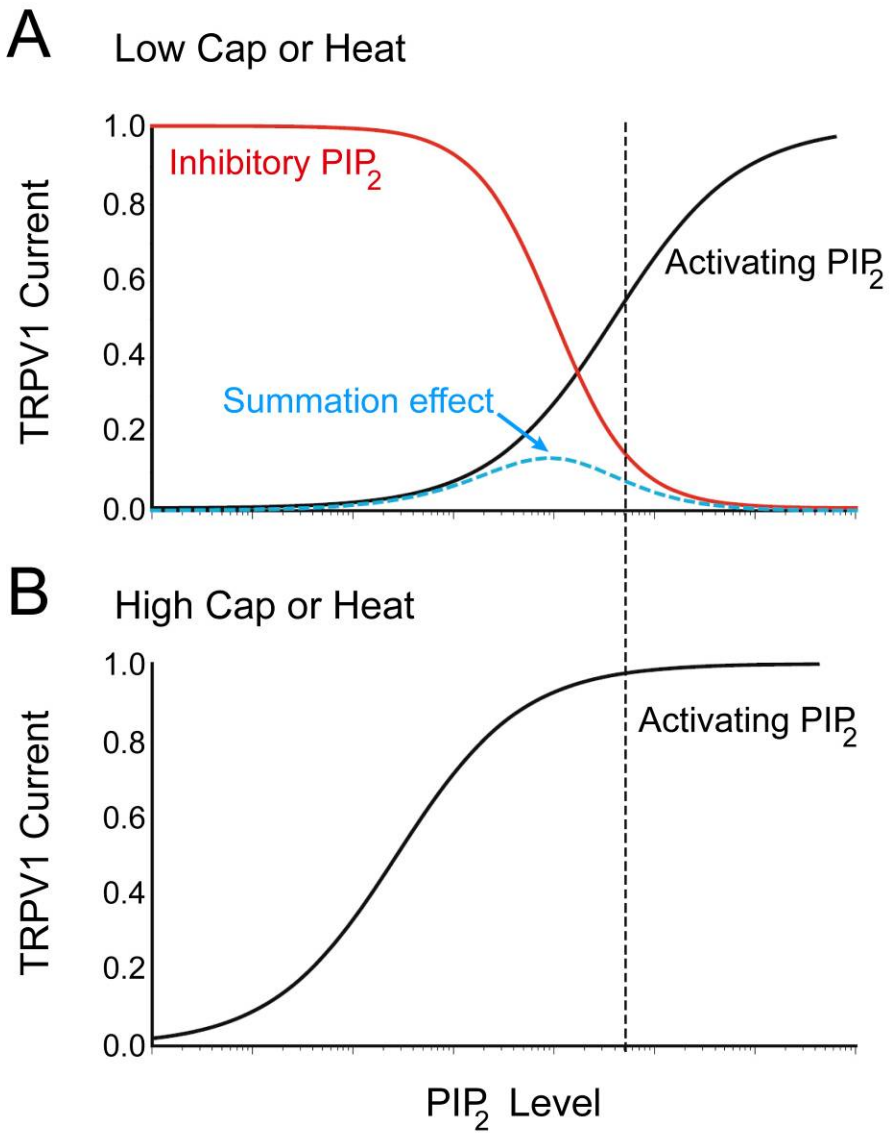

Figure 1.4. Model of PIP_2 regulation of TRPV1. PIP_2 has inhibitory and activating effects on TRPV1 activity depending on stimulus intensity. Rohacs et al. propose this model to explain these effects. (A) At low stimulus intensity, PIP_2 indirectly inhibits TRPV1 (red curve) but directly activates the channel (black curve). The sum of these two effects is the bell-shaped dose-response to PIP_2 levels (blue dashed line). If the resting PIP_2 levels in a cell are at the point of the vertical dashed line, a moderate decline in PIP_2 will lead to activation of the channel, while a more severe decline will lead to inhibition. An increase in PIP_2 will

lead to inhibition of the channel. (B) At high stimulus intensity, a depletion of any amount of PIP_2 will lead to inhibition of the channel, while increases may or may not affect channel activity. Regulation of the channel by PIP_2 is more complex than this model, but it provides a possible explanation for the observed co-existence of inhibitory and stimulatory effects of PIP_2 on TRPV1 activity. (Figure modified from Rohacs et al., 2008). (Back to text).

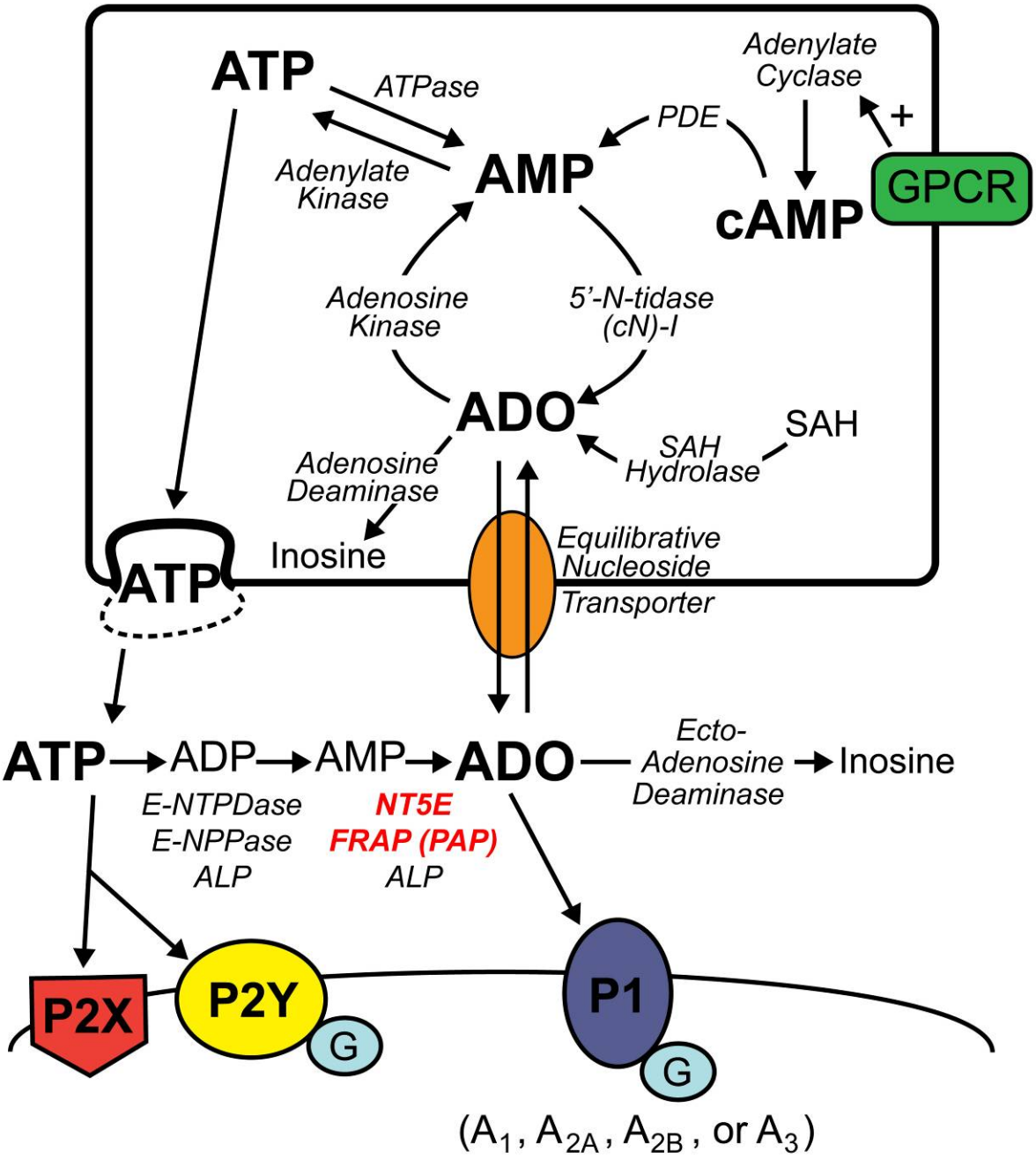

Figure 1.5. Intracellular and extracellular metabolism of adenosine. Inside the cell, adenosine (ADO) is made from ATP, cAMP, or SAH, while outside the cell it comes from release through equilibrative nucleoside transporters or catabolism of ATP. See text for details. PDE = Phosphodiesterase. (Figure modified from Sawynok and Liu, 2003). (Back to text)

Table 1.1. Prominent modulators of nociception

Molecule	Origin	Receptor(s)	Nociceptive Actions	References
Adenosine triphosphate (ATP)	Released from synaptic vesicles and damaged cells	$P2X_3$ $P2X_4$ $P2X_7$ $P2Y_{1,2}$	-Direct application evokes pain and excites C fibers -ATP-induced pain increased in response to PGE_1 and carrageenan -Important in development of neuropathic pain – activates spinal microglia	(Cook and McCleskey, 2002; Coutts et al., 1981; Hilliges et al., 2002; Ralevic and Burnstock, 1998; Sawynok et al., 2000; Stucky et al., 2004; Tsuda et al., 2003)
Bradykinin (BK)	Kininogen precursor proteins from activation of plasma or tissue kallikrein enzymes	B_2	-Acts directly to induce nociceptor firing through PKC activation -Sensitizes TRPV1 to induce thermal hyperalgesia -Stimulates production of pro-nociceptive molecules, including 12-HPETE and leukotriene B_4	(Calixto et al., 2004; Cesare et al., 1999a; McMahon et al., 2006; Premkumar and Ahern, 2000; Shin et al., 2002)
Histamine	Released from mast cells in response to substance P	H_{1-4}	-Excites polymodal visceral nociceptors -Potentiates nociceptor responses to BK and heat	(Koda et al., 1996; McMahon et al., 2006; Mizumura et al., 1995)
Nerve growth factor (NGF)	Increased synthesis and release from cells in inflamed tissues induced by growth factors and cytokines	TrkA TrkB TrkC	-Induces inflammatory pain responses -Sensitizes nociceptors and induces spontaneous firing activity -Sensitizes TRPV1 and TTX-resistant Na^+ channels -Induces release of histamine and serotonin from mast cells -Induces expression of cytokines in mast cells	(Bullock and Johnson, 1996; Chuang et al., 2001; Horigome et al., 1994; Kerr et al., 2001; McMahon, 1996; McMahon et al., 2006; Meyer et al., 2006)

Nitric oxide (NO)	From activation of spinal nNOS		-Important in the initiation and maintenance of inflammatory hyperalgesia -Production of cGMP from NO-sensitive guanylyl cyclase (NO-GC) and subsequent activation of pro-nociceptive cGMP-dependent protein kinase I (cGKI) -Activation of Cox enzymes and production of PGE_2 and PGI_2	(Handy and Moore, 1998; McMahon et al., 2006; Schmidtko et al., 2009; Toriyabe et al., 2004)
Prostaglandins (PGE_2, PGI_2)	Breakdown of arachidonic acid by Cox enzymes to PGH_2, then further synthesis to PGE_2 or PGI_2	$EP_{1, 3, 4}$ IP	-Sensitization of afferent neurons to noxious chemical, thermal, and mechanical stimuli -Reduces threshold for activation of TTX-resistant Na^+ channels -Increases intracellular cAMP -Increases neuronal excitability	(Birrell et al., 1991; England et al., 1996; Gold et al., 1996; Meyer et al., 2006; Mizumura et al., 1987)
Protease-activated receptor 2 (PAR2)	Activated by tryptase following tissue damage and inflammation	PAR2	-Produces prolonged thermal and mechanical hyperalgesia -Enhances TRPV1 activity through PKC and PKA	(Amadesi et al., 2004; Dai et al., 2004b; Kawabata et al., 2002; Kawabata et al., 2001; Meyer et al., 2006)
Protons (H^+)	Produced in response to tissue inflammation, hypoxia, and anoxia		-Causes prolonged activation of sensory nerves and produce sharp, stinging pain -Activates and sensitizes TRPV1 -Activation of pro-nociceptive acid sensing ion channels (ASICs) -Inhibits K^+ channels	(Baumann et al., 2004; Jones et al., 2004; Lindahl, 1962; Steen and Reeh, 1993; Sutherland et al., 2001; Tominaga et al., 1998)
Serotonin (5-HT)	Released from platelets and mast cells in inflamed tissue	$5\text{-}HT_{1B}$ $5\text{-}HT_{1D}$ $5\text{-}HT_{2A}$ $5\text{-}HT_{2B}$ $5\text{-}HT_{3B}$ $5\text{-}HT_4$	-Produces thermal hyperalgesia following inflammation -Reduces resting K^+ conductance -Increases TTX-resistant Na^+ channel activation	(Abbott et al., 1996; Cardenas et al., 1997; Nicholson et al., 2003; Todorovic and Anderson, 1990; Tokunaga et al., 1998)

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CHAPTER 2

Prostatic Acid Phosphatase Suppresses Pain by Generating Adenosine

ABSTRACT: Thiamine monophosphatase (TMPase, also known as fluoride-resistant acid phosphatase) is a classic histochemical marker of small-diameter dorsal root ganglia neurons. The molecular identity of TMPase is currently unknown. We found that TMPase is identical to the transmembrane isoform of prostatic acid phosphatase (PAP), an enzyme with unknown molecular and physiological functions. We then found that *PAP* knockout mice have normal acute pain sensitivity but enhanced sensitivity in chronic inflammatory and neuropathic pain models. In gain-of-function studies, intraspinal injection of PAP protein has potent antinociceptive, antihyperalgesic, and antiallodynic effects that last longer than the opioid analgesic morphine. PAP suppresses pain by functioning as an ecto-5′-nucleotidase. Specifically, PAP dephosphorylates extracellular adenosine monophosphate (AMP) to adenosine and activates A_1-adenosine receptors in dorsal spinal cord. Our studies reveal molecular and physiological functions for PAP in purine nucleotide metabolism and nociception and suggest a novel use for PAP in the treatment of chronic pain.

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2.1) Introduction

Painful and tissue-damaging stimuli are sensed by small-diameter nociceptive neurons located in the dorsal root ganglia (DRG) and trigeminal ganglia (Woolf and Ma, 2007) For nearly 50 years, it was known that many small-diameter DRG neurons expressed a histochemically identifiable acid phosphatase (Colmant, 1959), commonly referred to as fluoride-resistant acid phosphatase (FRAP) or thiamine monophosphatase (TMPase) (Dodd et al., 1983; Knyihar-Csillik et al., 1986). TMPase dephosphorylates diverse substrates, including the vitamin B_1 derivative thiamine monophosphate (TMP) and 5′-nucleotide monophosphates (Dodd et al., 1983; Sanyal and Rustioni, 1974; Silverman and Kruger, 1988a).

TMPase was intensively studied in the 1980s in an effort to determine its molecular identity and function. TMPase marks most nonpeptidergic DRG neurons, a subset of peptidergic DRG neurons, and unmyelinated axon terminals in lamina II of the dorsal spinal cord (Carr et al., 1990; Dalsgaard et al., 1984; Dodd et al., 1983; Hunt and Rossi, 1985; Knyihar-Csillik et al., 1986; Nagy and Hunt, 1982; Silverman and Kruger, 1988a). Since peptidergic and nonpeptidergic neurons are generally considered to be nociceptive (Woolf and Ma, 2007), these anatomical studies suggested that TMPase might function in nociception. Moreover, TMPase staining in lamina II of spinal cord is reduced or eliminated when peripheral nerves are damaged (Colmant, 1959; Shields et al., 2003; Tenser, 1985; Tenser et al., 1991). Ultimately, studies of TMPase waned when it was found that isolectin B4 (IB4) colocalized with TMPase and was an easier-to-use marker of nonpeptidergic neurons (Silverman and Kruger, 1988b). More importantly, the gene encoding TMPase was

never identified, making it impossible to study the molecular and physiological function of TMPase in sensory neurons.

In an attempt to identify the TMPase gene, Dodd and coworkers partially purified TMPase protein from rat DRG using chromatography (Dodd et al., 1983). The partially purified rat protein was inhibited by the nonselective acid phosphatase inhibitor L(+)-tartrate and was similar in molecular weight to the secretory isoform of human prostatic acid phosphatase (PAP, also known as ACPP), the only known isoform of PAP at the time (Ostrowski and Kuciel, 1994). These biochemical experiments hinted that TMPase might be secretory PAP (Dodd et al., 1983). However, subsequent studies using anti-PAP antibodies failed to immunostain small-diameter DRG neurons and their axon terminals in lamina II (i.e., the neurons and axons that contain TMPase) (Dodd et al., 1983; Silverman and Kruger, 1988a). As summarized by Silverman and Kruger in 1988, these data made it impossible to determine whether TMPase was PAP or some other enzyme.

In light of this unsolved question regarding the molecular nature of TMPase and the historical use of TMPase as a nociceptive neuron marker, we sought to definitively identify the TMPase gene and ascertain its function in nociception. Our experiments revealed that TMPase was a recently discovered transmembrane (TM) isoform of PAP (TM-PAP) (Quintero et al., 2007) and was not the secretory isoform of PAP. This molecular identification then allowed us to use modern molecular and genetic approaches to rigorously study the function of PAP/TMPase in nociceptive circuits. Using our *PAP* knockout mice, we found that deletion of PAP increased thermal hyperalgesia (increased pain sensitivity) and mechanical allodynia in animal models of chronic pain. Conversely, a single intraspinal injection of PAP protein had antinociceptive, antihyperalgesic, and antiallodynic effects that

lasted for up to 3 days, much longer than a single injection of the commonly used opioid analgesic morphine. Mechanistically, we found that PAP is an ectonucleotidase that dephosphorylates extracellular AMP to adenosine and requires A_1-adenosine receptors (A_1Rs) for antinociception.

PAP has been intensively studied for 70 years in the prostate cancer field (Gutman and Gutman, 1938). Despite decades of research, the molecular and physiological functions for PAP remained unknown. Our studies with pain-sensing neurons identify the in vivo substrate, the molecular mechanism, and the physiological function for this medically relevant protein. Moreover, we show that PAP functions in nociception. Considering that TM-PAP is expressed throughout the body (Quintero et al., 2007), PAP could regulate diverse physiological processes that are dependent on adenosine (Jacobson and Gao, 2006).

2.2) Materials and Methods

All procedures and behavioral experiments involving vertebrate animals were approved by Institutional Animal Care and Use Committees at the University of North Carolina at Chapel Hill and at the Universities of Oulu and Helsinki.

2.2.1) Molecular Biology

The full-length expression construct of PAP-transmembrane isoform (nt 64–1317 from GenBank accession # NM_207668) was generated by RT-PCR amplification, using C57BL/6 mouse trigeminal cDNA as template and Phusion polymerase. PCR products were cloned into pcDNA3.1 and completely sequenced. Isoform-specific in situ hybridization probes of PAP, secreted variant (nt 1544–2625 from GenBank accession # NM_019807), and

PAP, transmembrane variant (nt 1497–2577 from GenBank accession # NM_207668) were generated by PCR amplification, using C57BL/6 mouse genomic DNA as template and Phusion polymerase, then cloned into pBluescript-KS.

In situ hybridization was performed as described previously using digoxygenin-labeled antisense and sense (control) riboprobes (Dong et al., 2001). We confirmed that PAP was expressed in human DRG by performing RT-PCR with RNA from human DRG (Clontech) and primers that spanned three introns (exon 6 primer: 5′ ctttcaggattacatggccagg; exon 9 primer: 5′ cgtcaagtggcaagaagcatag).

2.2.2) Tissue Preparation

Adult male mice, 6–12 weeks of age, were sacrificed by cervical dislocation, decapitation, or pentobarbital overdose. Lumbar spinal cord and DRG (L4–L6) were dissected and then immersion fixed for 8 hr and 2 hr, respectively, in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4. Tissues were cryoprotected in 20% sucrose, 0.1 M phosphate buffer, pH 7.3 at 4°C for 24 hr, frozen in OCT, sectioned with a cryostat at 15–20 μm, and mounted on Superfrost Plus slides. Slides were stored at −20°C. Free-floating sections were sectioned at 30 μm and immediately stained.

2.2.3) Histology

Enzyme histochemistry was performed essentially as described by Shields et al., with modifications suggested by Silverman and Kruger (Shields et al., 2003; Silverman and Kruger, 1988a). Briefly, cells or tissue sections were washed twice with 40 mM Trizma-Maleate (TM) buffer, pH 5.6, then once with TM buffer containing 8% (w/v) sucrose.

Samples were then incubated at 37°C for 2 hr in TM buffer containing 8% sucrose (w/v), 6 mM thiamine monophosphate chloride or adenosine monophosphate (0.3 mM for tissue sections, 6 mM for HEK293 cells), and 2.4 mM lead nitrate. Lead nitrate must be made fresh immediately prior to use. To reduce nonspecific background staining, samples were washed once with 2% acetic acid for 1 min. Samples were then washed three times with TM buffer, developed for 10 s with 1% sodium sulfide, washed several times with PBS, pH 7.4, and mounted in Gel/Mount (Biomeda). When assaying HEK293 cells using TMP or AMP histochemistry, we stained duplicate samples with and without 0.1% Triton X-100 in the initial TM wash. Images were acquired using a Zeiss Axioskop and Olympus DP-71 camera.

Immunofluorescence was performed using antibodies and procedures essentially as described (Zylka et al., 2005), although we substituted high salt TBS + TX (50 mM Tris, 2.7% NaCl, 0.3% Triton X-100, pH 7.6) for PBS + TX in all wash and antibody incubation steps. Additional antibodies included 1:750 rabbit anti-CGRP (T-4032; Peninsula), 1:250 mouse anti-NeuN (MAB377, Chemicon), 1:300 guinea pig anti-P2X3 (GP10108, Neuromics), 1:1000 goat anti-VR1 (sc-12498, Santa Cruz), and 1:1000 rabbit anti-human PAP (Biomeda). We found that it was necessary to amplify the anti-PAP antibody signal by using secondary antibodies conjugated to biotin, then using either 1:250 streptavidin-Cy3 (Jackson) or the Tyramide Signal Amplification kit (New England Nuclear, following manufacturer's protocol). Images were obtained using a Leica TCS-NT confocal microscope.

2.2.4) Injections and Drugs

For intrathecal drug delivery, 5 μL was injected into unanesthetized mice using the direct lumbar puncture method (Fairbanks, 2003). Human PAP (Sigma, P1774, 100 U/ml)

was dialyzed against 0.9% saline using Slide-A-Lyzer Mini dialysis units (Pierce, 69576) for 4 hr at 4°C. After dialysis, samples were diluted in 0.9% saline to a final concentration of 1.3 mg/ml (50 U/ml) and stored at −80°C. S-hPAP was heat-inactivated by incubating at 65°C for 40 min. Bovine prostatic acid phosphatase (bPAP, Sigma, P6409) was dissolved (aided by sonication) in 0.9% saline to a final concentration of 30 mg/ml (0.3 U/ml). Enzyme activity was quantified using the EnzChek Phosphatase Assay Kit (Invitrogen, E12020) following the manufacturer's protocol. Bovine serum albumin (BSA, Sigma, A3912) was dissolved in 0.9% saline to a final concentration of 1.3 mg/ml. Recombinant bovine alkaline phosphatase was purchased from Sigma (P8361, expressed in *Pichia pastoris*, >4000 U/mg protein). Morphine sulfate (Sigma, M8777) and N^6-cyclopentyladenosine (Sigma, C8031; 10 mM stock in dimethylsulfoxide; DMSO) were diluted into 0.9% saline. 8-cyclopentyl-1, 3-dipropylxanthine (C101, Sigma) was dissolved in 0.9% saline containing 5% DMSO, 1.25% 1 M NaOH, and injected i.p.

2.2.5) Behavior

$Pap^{-/-}$ and $A_1R^{-/-}$ mice were backcrossed to C57BL/6 mice (Jackson) for 10 and 12 generations, respectively. Isogenic wild-type mice were then derived from the $Pap^{-/-}$ line and used as wild-type controls. C57BL/6 male mice were purchased from Jackson Laboratories for all behavioral experiments involving PAP protein injections. Unless indicated otherwise, male mice, 2–4 months old, were used for all behavioral experiments. All mice were acclimated to the testing room, equipment, and experimenter for 1–3 days before behavioral testing. The experimenter was blind to genotype and drug treatment during behavioral testing.

Thermal sensitivity was measured by heating one hindpaw with a Plantar Test apparatus (IITC) following the Hargreaves method (Hargreaves et al., 1988). The radiant heat source intensity was calibrated so that a paw-withdrawal reflex was evoked in ~10 s., on average, in wild-type C57BL/6 mice. Cutoff time was 20 s. One measurement was taken from each paw per day to determine paw-withdrawal latency (with the exception of our morphine and CPA dose-response experiments, which required multiple measurements per day). To perform the tail-immersion assay, mice were gently restrained in a towel and the distal one-third of the tail was immersed in 46.5°C or 49°C water. Latency to withdrawal the tail was measured once per mouse. For the hot plate test, mice were placed on a metal surface heated at 52°C and latency to jump, lick paws, or shake paws was measured. Mechanical sensitivity was measured using semiflexible tips attached to an electronic von Frey apparatus (IITC) as described elsewhere (Cunha et al., 2004; Inoue et al., 2004). Three measurements were taken from each paw (separated at 10 min intervals) then averaged to determine paw-withdrawal threshold in grams.

To induce inflammatory pain, 20 μl complete Freund's adjuvant (CFA, from Sigma or MP Biomedicals) was injected into one hindpaw, centrally beneath glabrous skin, with a 30G needle. The spared nerve injury (SNI) model of neuropathic pain was performed as described (Shields et al., 2003)

2.3) Results

2.3.1) Prostatic Acid Phosphatase Is TMPase in Dorsal Root Ganglia Sensory Neurons

In rats, mice, and humans, PAP is expressed as a secreted protein or as a type 1 transmembrane (TM) protein, with the catalytic acid phosphatase domain localized

extracellularly (Figure 2.1A) (Quintero et al., 2007; Roiko et al., 1990; Vihko, 1979). The secretory isoform has been used as a diagnostic marker for prostate cancer for nearly 70 years, whereas the TM isoform was only recently discovered (Gutman and Gutman, 1938; Quintero et al., 2007). To determine whether either PAP isoform is expressed in DRG, we performed in situ hybridization with isoform-specific antisense riboprobes. These experiments revealed that TM-PAP was expressed in a subset of small-diameter DRG neurons (Figure 2.1B), while the secretory isoform was expressed at low-to-undetectable levels (Figure 2.1C). Importantly, TM-PAP is localized to the plasma membrane and vesicular membranes, just like TMPase (Csillik and Knyihar-Csillik, 1986; Quintero et al., 2007). We also found that PAP was expressed in human DRG using RT-PCR and intron-spanning primers (data not shown), consistent with localization of TMPase to small-diameter human DRG neurons (Silverman and Kruger, 1988a).

To directly test whether PAP had TMPase histochemical activity, we overexpressed mouse TM-PAP in HEK293 cells, then stained these cells using TMP histochemistry. Cells transfected with TM-PAP were heavily stained when the plasma membrane was left intact (Figure 2.2A), indicating that TM-PAP can dephosphorylate TMP extracellularly. TMPase staining was even greater when the plasma membrane was permeabilized with detergent (Figure 2.3H). In contrast, control cells transfected with empty vector were not stained (Figure 2.2B). Two additional phosphatases (soluble acid phosphatase 1 [ACP1] and placental alkaline phosphatase) lacked TMPase activity (Figure 2.3).

DRG neurons express at least eight different acid phosphatase genes (M.J.Z, unpublished data), any one of which could be TMPase. To determine whether PAP was the only enzyme in sensory neurons capable of dephosphorylating TMP, we analyzed DRG and

spinal cord tissues from *PAP*$^{\Delta 3/\Delta 3}$ (henceforth referred to as *Pap*$^{-/-}$) knockout mice (P.V. et al., abstract from Proceedings of the AACR, 2005, 96th Annual Meeting, Anaheim, CA). In these mice, deletion of exon 3 causes complete loss of secretory and transmembrane PAP catalytic activity (P.V. et al., abstract from Proceedings of the AACR, 2005). Strikingly, TMP histochemical staining of DRG neurons and axon terminals in spinal cord was abolished in *Pap*$^{-/-}$ mice (Figures 2.2C–2.2F). Absence of TMP staining in *Pap*$^{-/-}$ mice was not due to developmental loss of DRG neurons, as wild-type and *Pap*$^{-/-}$ mice had equivalent numbers of P2X3-expressing neurons relative to all NeuN^{+} neurons in lumbar ganglia (43.4% ± 1.9% verses 42.4% ± 1.9% (SEM); 1500 NeuN^{+} neurons counted per genotype). $P2X_3$ is an ATP-gated ion channel that is colocalized with PAP (see below). Moreover, loss of TMPase staining in the spinal cord was not due to loss of axon terminals in the dorsal horn (Figure 2.4). These gain- and loss-of-function experiments conclusively prove that TMPase in small-diameter DRG neurons is the transmembrane isoform of PAP.

In addition, by combining immunofluorescence and TMP histochemistry, we observed colocalization between PAP and TMPase in DRG neurons (Figures 2.5A–2.5C) and in axon terminals in lamina II of the spinal cord (Figures 2.5D–2.5F). This anti-PAP antibody did not stain DRG or spinal cord sections from *Pap*$^{-/-}$ mice, confirming antibody specificity. Finally, upon finding that PAP was TMPase, we reanalyzed two published microarray data sets that measured changes in gene expression in DRG following peripheral nerve injury (Costigan et al., 2002; Davis-Taber and Scott, 2006). In both studies, PAP was one of the most heavily downregulated genes. This is consistent with the fact that TMPase histochemical activity is greatly reduced in DRG and dorsal horn after peripheral nerve

injury (Colmant, 1959; Csillik and Knyihar-Csillik, 1986; Shields et al., 2003; Tenser, 1985; Tenser et al., 1991).

2.3.2) PAP Is Primarily Expressed in Nonpeptidergic DRG Neurons

TMPase was previously localized to nonpeptidergic DRG neurons and a small number of peptidergic neurons (Carr et al., 1990; Dalsgaard et al., 1984; Hunt and Mantyh, 2001; Nagy and Hunt, 1982; Silverman and Kruger, 1988b). To show that PAP had a similar distribution and to identify additional proteins that were colocalized with PAP, we performed double-label immunofluorescence with our anti-PAP antibody and various sensory neuron markers. Cell counts from confocal images revealed that virtually all nonpeptidergic DRG neurons, as defined by the markers IB4, *Mrgprd-EGFPf*, and P2X3, coexpressed PAP (Figures 2.6A–2.6I and Table 2.1). Moreover, PAP^+ axons terminated in lamina II of spinal cord in association with nonpeptidergic neuron markers (Figures 2.7A–2.7F). In contrast, a smaller percentage (17.1%) of peptidergic $CGRP^+$ neurons (n = 1364 cells counted) expressed PAP (Figures 2.6J–2.6L and Table 2.1), and there was minimal overlap between PAP^+ and peptidergic ($CGRP^+$) axon terminals in spinal cord (Figures 2.7G–2.7I). Finally, 19.1% ± 1.3% of PAP^+ neurons expressed the capsaicin and noxious heat receptor TRPV1 (Figures 2.6M–2.6O). Taken together, these confocal imaging studies revealed that PAP was preferentially expressed in nonpeptidergic, presumably nociceptive, DRG neurons.

2.3.3) Chronic Pain-Induced Thermal Hyperalgesia and Mechanical Allodynia Are Enhanced in *Pap* Knockout Mice

PAP was generally thought to function only in the prostate (Ostrowski and Kuciel, 1994). However, our expression data suggested that PAP might also function in nociceptive neurons. To evaluate pain-related functions for PAP, we tested age-matched wild-type C57BL/6 and *Pap*$^{-/-}$ male mice (backcrossed to C57BL/6 for ten generations) using acute and chronic pain behavioral assays. We found no significant differences between genotypes using a measure of mechanical sensitivity (electronic von Frey) or several different measures of acute noxious thermal sensitivity (Table 2.2). In contrast, *Pap*$^{-/-}$ mice showed significantly greater thermal hyperalgesia and mechanical allodynia relative to wild-type mice in the complete Freund's adjuvant (CFA) model of chronic inflammatory pain (Figures 2.8A and 2.8B). In addition, *Pap*$^{-/-}$ mice showed significantly greater thermal hyperalgesia in the spared nerve injury (SNI) model of neuropathic pain (Figures 2.8C and 2.8D) (Shields et al., 2003).

2.3.4) PAP Has Potent and Long-Lasting Antinociceptive Properties

Since deletion of PAP enhanced sensitivity in two different models of chronic pain, we hypothesized that excess PAP should have the opposite effect and reduce pain. To test this, we took advantage of the fact that secretory PAP protein is commercially available and has the same N-terminal catalytic region as TM-PAP (Figure 2.1A). We injected wild-type mice intrathecally (i.t.) into the lumbar region of spinal cord with pure human (S-h)PAP protein (the secretory isoform). Control mice were injected i.t. with an equivalent amount of heat-denatured, and hence phosphatase-inactive, S-hPAP protein. In all cases, we determined that S-hPAP was active or inactive using a sensitive fluorometric-based phosphatase assay (see Materials and Methods). We then measured noxious thermal and mechanical sensitivity

before (baseline, BL) and after S-hPAP injections (Figures 2.9A and 2.9B). Six hours after i.t. injection of S-hPAP, paw-withdrawal latency to a noxious thermal stimulus significantly increased relative to controls and remained elevated for 3 days (Figure 2.9A). This antinociceptive effect was dose dependent (Figure 2.10) and required PAP catalytic activity (Figure 2.9A). Active S-hPAP did not alter mechanical sensitivity (Figure 2.9B) nor did it cause paralysis or sedation. This long-lasting antinociceptive effect was species conserved, as a single i.t. injection of bovine (b)PAP also increased thermal withdrawal latency for 2 days but had no effect on mechanical sensitivity (Figure 2.11). Finally, i.t. injection of an unrelated protein (bovine serum albumin) or large quantities of a different secreted phosphatase (bovine alkaline phosphatase) did not alter thermal or mechanical sensitivity (Figures 2.11 and 2.12).

We next used the same behavioral assay to compare PAP antinociception to the commonly used opioid analgesic morphine. We found that PAP and morphine antinociception were similar in magnitude following a single i.t. injection (40.8% ± 3.3% versus 62.2% ± 9.9% increase above baseline at the highest doses, respectively) but that PAP antinociception lasted much longer than morphine (3 days verses 5 hr at the highest doses, respectively; Figures 2.10 and 2.13). Similarly, Grant and colleagues found that the same high dose of morphine (50 μg, i.t., single injection) lasted 4.6 ± 1.0 hr in mice (Grant et al., 1995).

We next evaluated the extent to which S-hPAP affected hyperalgesia and allodynia in the CFA model of inflammatory pain and the SNI model of neuropathic pain. For both chronic pain assays, we used the uninjured paw as control. Strikingly, in both chronic pain models, a single i.t. injection of active S-hPAP was antihyperalgesic *and* antiallodynic in the

inflamed/injured paw (Figures 2.9C–2.9F). As before, a single injection was effective for several days, and phosphatase activity was required for these antinociceptive effects.

Since *Pap*$^{-/-}$ mice showed enhanced hyperalgesia and allodynia in the CFA inflammatory pain model (Figures 2.8A and 2.8B), we next tested whether S-hPAP could rescue these enhanced thermal and mechanical phenotypes in *Pap*$^{-/-}$ mice. We found that i.t. injection of S-hPAP increased thermal withdrawal latency in the control paw of *Pap*$^{-/-}$ mice to the same extent as wild-type mice (Figure 2.14A, blue lines). This demonstrated that *Pap*$^{-/-}$ mice were competent to respond to acute increases in PAP activity. Strikingly, injection of S-hPAP rescued the thermal and mechanical inflammatory pain phenotype in the inflamed paw of *Pap*$^{-/-}$ mice (Figures 2.14A and 2.14B, compare red lines where PAP was injected to black lines where inactive PAP was injected). Importantly, these data also suggest that localized, spinal injection of S-hPAP can rescue the behavioral deficit caused by deletion of PAP throughout the animal.

2.3.5) PAP Suppresses Pain by Generating Adenosine, a Known Analgesic in Mammals

The antinociceptive effects of PAP require catalytic activity. This suggested PAP might generate, via dephosphorylation, a molecule that regulates nociceptive neurotransmission in the spinal cord. PAP and TMPase can dephosphorylate many different substrates (Dziembor-Gryszkiewicz et al., 1978; Sanyal and Rustioni, 1974; Silverman and Kruger, 1988b; Vihko, 1978). We focused on AMP because dephosphorylation of AMP produces adenosine—a molecule that inhibits nociceptive neurotransmission in spinal cord slices and has well-studied analgesic properties in mammals (Li and Perl, 1994; Liu and Salter, 2005; Post, 1984; Sawynok, 2007).

At the time we began our studies, there was no direct proof that PAP or TMPase could generate adenosine from AMP. Instead, production of adenosine was inferred by measuring production of inorganic phosphate (Vihko, 1978). To directly test whether PAP could generate adenosine from AMP and other adenine nucleotides, we incubated PAP with 1 mM AMP, ADP, or ATP at pH 7.0 for 4 hr. We then detected adenine nucleotides and adenosine using high-performance liquid chromatography (HPLC) and UV absorbance (Lazarowski et al., 2004). These studies revealed that PAP can rapidly dephosphorylate AMP and, to a much lesser extent, ADP to adenosine (Figures 2.15A and 2.15B). Importantly, no unexpected peaks were seen in the chromatograms (Figure 2.15B, data not shown), ruling out the possibility that PAP had additional hydrolytic activities toward nucleotides.

Next, we tested the extent to which PAP could dephosphorylate extracellular AMP in HEK293 cells, DRG neurons, and spinal cord using AMP enzyme histochemistry. HEK293 cells transfected with TM-PAP were heavily stained, whereas control cells were not (Figures 2.15C and 2.15D), highlighting that TM-PAP dephosphorylates extracellular AMP and hence has ecto-5′-nucleotidase activity. In addition, small-diameter DRG neurons from wild-type mice were intensely stained while large-diameter neurons had weak granular cytoplasmic staining. In contrast, only weak granular staining was present in DRG neurons from *PAP*$^{-/-}$ mice (Figures 2.15E and 2.15F). These data indicate that PAP is the predominant ecto-5′-nucleotidase on the soma of small-diameter neurons. Finally, AMP histochemical staining of axon terminals in lamina II was reduced in *PAP*$^{-/-}$ relative to wild-type mice, but was not eliminated (Figures 2.15G and 2.15H). This indicates that PAP is one of perhaps many enzymes in spinal cord with the ability to dephosphorylate AMP to adenosine.

Adenosine mediates antinociception through G_i-coupled A_1-adenosine receptors (A_1Rs) (Lee and Yaksh, 1996; Sawynok, 2007). To directly test whether A_1Rs were required for PAP antinociception, we next i.t. injected S-hPAP into wild-type C57BL/6 and A_1-adenosine receptor knockout mice ($A_1R^{-/-}$, *Adora1*$^{-/-}$; backcrossed to C57BL/6 mice for 12 generations), then measured noxious thermal and mechanical sensitivity (Hua et al., 2007; Johansson et al., 2001). Strikingly, S-hPAP increased thermal paw-withdrawal latency for 3 days in wild-type mice but was without effect in $A_1R^{-/-}$ mice (Figure 2.16A). Similarly, bPAP increased paw-withdrawal latency to the noxious thermal stimulus in wild-type mice but had no effect in $A_1R^{-/-}$ mice (Figure 2.17). As expected, S-hPAP did not affect mechanical sensitivity in uninjured animals (Figure 2.16B).

We next tested wild-type and $A_1R^{-/-}$ mice using the CFA chronic inflammatory pain model and the SNI neuropathic pain model. Reproducing previous findings (Wu et al., 2005), $A_1R^{-/-}$ mice showed greater thermal hyperalgesia compared to wild-type mice after CFA injection and after nerve injury (but before PAP injection; Figures 2.16C and 2.16E). Following i.t. injection of S-hPAP, thermal and mechanical thresholds increased in the inflamed/injured paws of wild-type mice but not in $A_1R^{-/-}$ mice (Figures 2.16C–2.16F). Likewise, the selective A_1R antagonist 8-cyclopentyl-1, 3-dipropylxanthine (CPX; 1 mg/kg, i.p.) transiently reversed the antinociceptive effects of S-hPAP in control and inflamed hindpaws (Figure 2.18). Conversely, injection (i.t.) of the selective A_1R agonist N^6-cyclopentyladenosine (CPA) into wild-type mice produced dose-dependent increases in paw-withdrawal latency to our thermal stimulus (Figure 2.19), similar to i.t. S-hPAP. However, unlike S-hPAP, CPA had short-term effects (lasting hours not days) and CPA caused transient paralysis at the two highest doses. When taken together, our results demonstrate that

the antinociceptive effects of PAP are due to generation of adenosine followed by activation of A_1Rs. Moreover, our data demonstrate an in vivo function for PAP as an ectonucleotidase.

2.4) Discussion

For 70 years, PAP was thought to be a secreted protein found only in the prostate and was used as a diagnostic marker for prostate cancer (Gutman and Gutman, 1938; Ostrowski and Kuciel, 1994). Despite years of research, little was known about how PAP functioned in vivo at a mechanistic level or which PAP substrate was most biologically relevant. In biochemical assays, PAP can dephosphorylate diverse substrates, including β-glycerophosphate, lysophosphatidic acid, phospho-amino acids, and 5′-nucleotides (Dziembor-Gryszkiewicz et al., 1978; Li et al., 1984; Porvari et al., 1994; Tanaka et al., 2004; Vihko, 1978).

In our efforts to solve an old and unanswered question in the pain field, we found that PAP was expressed in nociceptive neurons, was antinociceptive, and functioned as an ectonucleotidase. Importantly, we found that the in vivo effects of PAP were eliminated by deletion of one gene, the A_1-adenosine receptor. This makes it unlikely that PAP suppresses pain by generating any other molecules besides adenosine. Moreover, the in vivo effects of PAP on acute and chronic pain mimic the effects of i.t. adenosine and A_1R agonists (Figure 2.19) (Liu and Salter, 2005; Sawynok, 2007). Notably, both PAP and adenosine receptor agonists have antiallodynic and antihyperalgesic properties in animal models of inflammatory and neuropathic pain, both have a long (>24 hr) duration of action after a single i.t. injection, and both lose their ability to suppress pain in $A_1R^{-/-}$ mice or following injection of A_1R antagonists (Belfrage et al., 1999; Cui et al., 1997; Eisenach et al., 2002;

Gomes et al., 1999; Johansson et al., 2001; Lavand'homme and Eisenach, 1999; Lee and Yaksh, 1996; Maione et al., 2007; Poon and Sawynok, 1998). In addition, both $A_1R^{-/-}$ and $PAP^{-/-}$ mice show enhanced thermal hyperalgesia, but not enhanced allodynia, in neuropathic pain models (Figures 2.8C and 2.8D) (Wu et al., 2005). This shared modality-selective phenotype further supports our conclusion that endogenous PAP works via A_1R activation. Although our studies were focused on nociceptive neurons, PAP is expressed in many other tissues (Quintero et al., 2007) and thus could function as an ectonucleotidase throughout the body.

2.4.1) PAP Has Potent and Long-Lasting Antinociceptive Effects when Compared to Opioid Analgesics

Morphine and other opioids are powerful analgesics but have side effects that limit their long-term use. We found that a single i.t. injection of S-hPAP (250 mU) produced an increase in paw-withdrawal latency of 40.8% ± 3.3% (relative to baseline; n = 74 mice) in the Hargreaves test (Figure 2.10C) and reproducibly lasted for 3 days (Figure 2.9, Figure 2.14 and Figure 2.16, 2.10A, and 2.18). Using the same behavioral test, we found that 1 μg and 10 μg of morphine produced an increase in paw-withdrawal latency of 24.9% ± 5.3% and 55.9% ± 13.7%, respectively, but lasted 1–4 hr in mice (Figure 2.13). Similarly, others found that 1 μg and 10 μg of morphine (i.t., single dose) produced an increase in paw-withdrawal latency of 36% and 60%, respectively (Dirig and Yaksh, 1995), that lasted hours in rats (Nishiyama et al., 2000; Zhang et al., 2005b). Higher doses of i.t. morphine cause motor impairment and death (Figure 2.13) (Dirig and Yaksh, 1995; Grant et al., 1995; Nishiyama et al., 2000). Although high doses of A_1R agonists also cause motor impairment (Figure 2.19) (Sawynok,

2007), we found no such side-effects at the highest dose of PAP tested. These comparisons reveal that the magnitude of PAP and morphine antinociception is similar; however, PAP antinociception lasts substantially longer. In fact, using area under the curve (AUC) measurements that integrate magnitude of antinociception over time, the 250 mU dose of S-hPAP is eight times more effective than the highest dose of morphine tested (Figures 2.10B and 2.13B). These long-lasting and A_1R-dependent antinociceptive effects of PAP are supported by previous studies showing that adenosine produces long-duration (>24 hr) analgesia in humans and rodents (Belfrage et al., 1999; Eisenach et al., 2002; Lavand'homme and Eisenach, 1999). Finally, we found that PAP antinociception could be *transiently* inhibited with an A_1R antagonist (Figure 2.18). This suggests that PAP is stable in spinal cord following injection and is capable of producing adenosine for days. Likewise, PAP has a very long (11.7 day) half-life in blood (Vihko et al., 1982).

2.4.2) PAP Is an Ectonucleotidase in Nociceptive Circuits

Nucleotides like ATP and ADP play key roles in pain mechanisms (Burnstock, 2007; Sawynok, 2007; Tozaki-Saitoh et al., 2008). Nucleotides are released extracellularly by stimulated sensory neurons and activate purinergic P2X and P2Y receptors on neurons and microglia. Activation of these receptors facilitates neurotransmission, sensitizes neurons, and causes pain. The excitatory effects of extracellular nucleotides can be terminated by several membrane-bound and, in some cases, secreted ectonucleotidases. These ectonucleotidases dephosphorylate extracellular ATP, ADP, and AMP to adenosine (Zimmermann, 2006). While ATP has excitatory effects and causes pain, adenosine has inhibitory effects and suppresses pain (Nakagawa et al., 2007; Sawynok, 2007).

One or more ectonucleotidases were known to exist in nociceptive circuits (Nagy and Daddona, 1985; Scott, 1967; Suran, 1974). Considering the key roles nucleotides and adenosine play in pain mechanisms, it is surprising to note that none of these ectonucleotidases have been molecularly identified. Using electrophysiological approaches, two groups found that application of ATP, ADP, or AMP inhibited postsynaptic neurons in the dorsal spinal cord indirectly via metabolic conversion to adenosine (Li and Perl, 1995; Salter and Henry, 1985). Likewise, Patterson and colleagues used indwelling microprobes and found that adenosine was metabolically generated in vivo when the dorsal spinal cord was perfused with AMP (Patterson et al., 2001). Dephosphorylation of AMP to adenosine was partially blocked by coperfusion with α,β-methylene-ADP (Patterson et al., 2001), an inhibitor of ecto-5′-nucleotidase (CD73). This enzyme has not yet been molecularly characterized in DRG neurons or spinal cord.

Our studies clearly show that PAP is expressed in small-diameter DRG neurons, that PAP dephosphorylates AMP to adenosine in vitro, in heterologous cells, in DRG neurons, and in lamina II of the spinal cord, and that PAP antinociception requires A_1Rs. To our knowledge, none of the known ectonucleotidases (Zimmermann, 2006) have been studied at this level of detail in nociceptive circuits. Collectively, our studies define PAP as an ectonucleotidase in nociceptive circuits.

2.4.3) PAP Is Well Localized to Modulate A_1-Adenosine Receptors in Spinal Cord Lamina II

Our data highlight a close functional relationship between PAP and A_1Rs. This raises the question of where PAP acts to modulate A_1Rs and nociceptive behaviors. Within the

spinal cord, A_1Rs are concentrated in lamina II, particularly on postsynaptic neurons that are in close contact with $IB4^+$ axon terminals, but not in close contact with $CGRP^+$ axon terminals (Schulte et al., 2003). A_1Rs are also found presynaptically in small- to medium-diameter DRG neurons, and possibly in the axon terminals of these neurons (based on accumulation of A_1Rs proximal to dorsal root ligature) (Schulte et al., 2003). In addition, A_1R activation inhibits presynaptic glutamate release primarily from unmyelinated terminals and inhibits postsynaptic neurons in the substantia gelatinosa (lamina II) of spinal cord (Lao et al., 2001; Li and Perl, 1994; Patel et al., 2001).

Considering that virtually all PAP^+ neurons and axons are $IB4^+$ (Table 2.1) and terminate in lamina II (Figure 2.7), this makes PAP well localized to generate extracellular adenosine and modulate A_1Rs on presynaptic terminals and on postsynaptic neurons in the IB4-binding region of lamina II. In addition, PAP (TMPase) is enriched on the presynaptic membranes of DRG neurons at the level of electron microscopy (Knyihar-Csillik et al., 1986) and has a broad pH optimum (pH 3–8) (Van Etten, 1982), making PAP capable of generating adenosine locally at synapses.

Further support for a central site of action comes from the fact that dephosphorylation of extracellular AMP in lamina II is reduced in $Pap^{-/-}$ mice and PAP is antinociceptive when injected intraspinally. Moreover, central injection of PAP can rescue behavioral deficits caused by deletion of PAP throughout the animal (Figure 2.14).

Although our data clearly support a central mechanism of action, we cannot exclude the possibility that PAP might also generate adenosine peripherally to mediate antinociception. Peripheral administration of adenosine has long-lasting antinociceptive and analgesic effects in rodents and humans, just like central administration (Hayashida et al.,

2005; Sawynok, 2007). We detected PAP on axons in the dermis of the skin; however, our antibody was not sensitive enough to detect PAP on axon terminals in epidermis (B.T-B. and M.J.Z, unpublished). Moreover, others found that TMPase (PAP) accumulated proximal to a ligature of the sural nerve, suggesting that PAP is transported peripherally (McMahon and Moore, 1988).

2.4.4) Physiological Function of PAP throughout the Body—Insights from Pain-Sensing Neurons

In prostate, PAP is thought to function as a tumor suppressor. Notably, prostate cancer cell growth rate is reduced when secretory S-hPAP (referred to as "cellular" PAP by the authors) is overexpressed (Lin et al., 1992; Meng and Lin, 1998; Veeramani et al., 2005). Conversely, deletion of PAP (secreted and TM isoforms) in mice leads to prostate hyperplasia followed by prostate cancer (P.V. et al., abstract from Proceedings of the AACR, 2005). The mechanism by which PAP mediates growth suppression is, at present, unclear. Correlative data from Lin and colleagues suggests that secretory ("cellular") PAP regulates growth directly by dephosphorylating phosphotyrosine residues in the cytoplasmic tail of ErbB2 (Veeramani et al., 2005). This direct mechanism seems unlikely, particularly since the cytoplasmic tail of ErbB2 is not accessible to the active site of secretory PAP (which is located extracellularly (Figure 2.1A) and in the lumen of vesicles). Instead, PAP could *indirectly* regulate proliferation by generating adenosine.

In support of this, there are four adenosine receptor subtypes, with A_1 and A_3 coupled to inhibitory G_i-proteins and A_{2a} and A_{2b} coupled to stimulatory G_q and G_s proteins (Jacobson and Gao, 2006). PAP could differentially modulate intracellular signaling

depending upon which adenosine receptor subtypes are expressed by cells. Notably, A_3-adenosine receptors are found on prostate cancer cells and A_3-agonists inhibit the growth of these cells (Fishman et al., 2003).

Adenosine regulates many other physiological processes besides pain and cancer, including anxiety, inflammation, blood pressure, pulmonary function, and renal function (Jacobson and Gao, 2006). TM-PAP is expressed throughout the body (Quintero et al., 2007). As such, TM-PAP could regulate diverse physiological processes that are dependent on adenosine. Finally, our study overturns the long-held belief that PAP is a generic “acid phosphatase” by discovering a specific in vivo function for PAP as an ectonucleotidase.

2.5) Figures and Tables

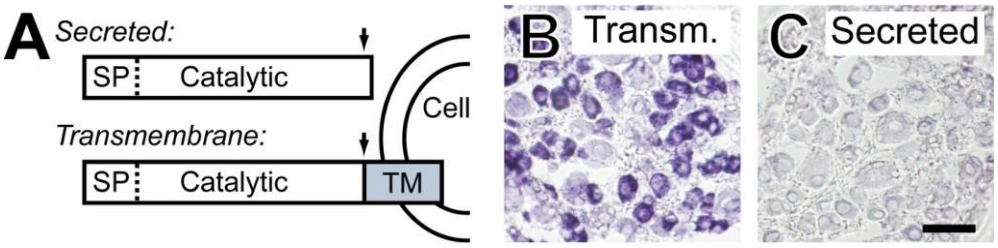

Figure 2.1. DRG neurons express the transmembrane isoform of PAP. (A) Secreted and transmembrane isoforms of PAP. Both isoforms have identical N-terminal regions, including the signal peptide (SP) and extracellular catalytic acid phosphatase domain. Alternative splicing at the last intron-exon junction (arrow) results in the inclusion or exclusion of a transmembrane (TM) domain. (B, C) *In situ* hybridization of mouse lumbar DRG with riboprobes complimentary to the unique 3' untranslated regions of (B) the transmembrane isoform or (C) the secreted isoform. Scale bar: 50 μm in (B), (C). (Back to text)

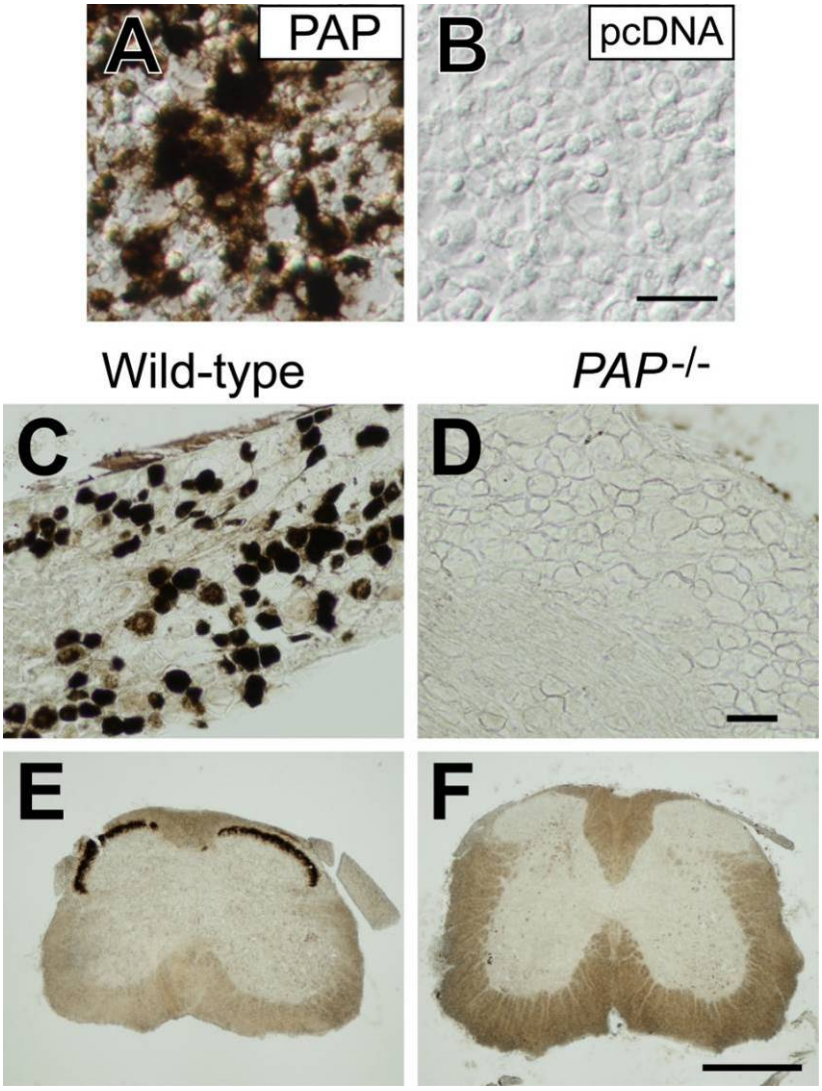

Figure 2.2. PAP dephosphorylates TMP in HEK 293 cells and nociceptive circuits. (A) HEK 293 cells were transfected with a mouse TM-PAP expression construct or (B) with empty pcDNA3.1 vector and then stained using TMP histochemistry. The plasma membrane was not permeabilized so that extracellular acid phosphatase activity could be assayed. (C-D) Lumbar DRG and (E-F) spinal cord from wild-type and *Pap*$^{-/-}$ adult mice stained using TMP histochemistry. Identical results were obtained from 14 additional mice of each genotype. TMP (6 mM) was used as substrate and buffer pH was 5.6 in all panels. Scale bar: 50 μm in (A-D); 500 μm in (E), (F). (Back to text)

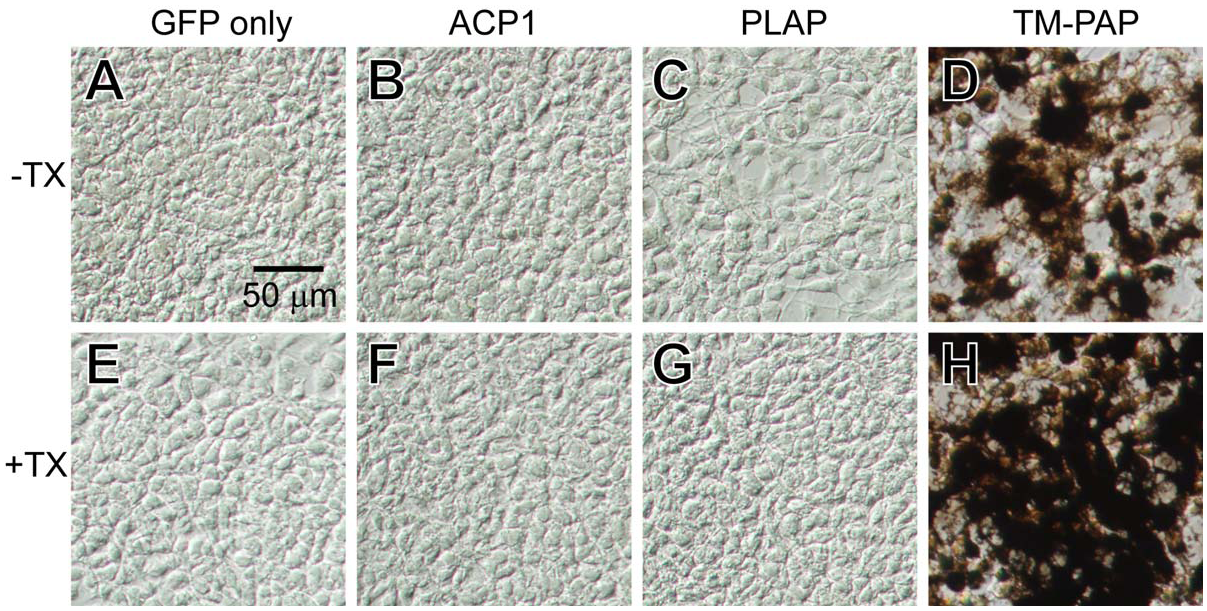

Figure 2.3. TM-PAP is the only phosphatase tested with TMPase activity. HEK 293 cells were transfected with full-length expression constructs for (A, E) GFP, (B, F) mouse soluble acid phosphatase 1 (ACP1), (C, G) human placental alkaline phosphatase (PLAP) or (D, H) mouse TM-PAP. The plasma membrane (A-D) was left intact (-TX) or (E-H) was permeabilized with the detergent Triton X-100 (+TX). Extracellular phosphatase activity is detectable in the absence of detergent whereas extracellular and intracellular phosphatase activity is detectable when cells are treated with detergent. The image in panel D was also shown in Figure 2.2A. All samples were stained using TMP histochemistry (6 mM TMP, pH 5.6). Scale bar: 50 µm in A-H. (Back to text)

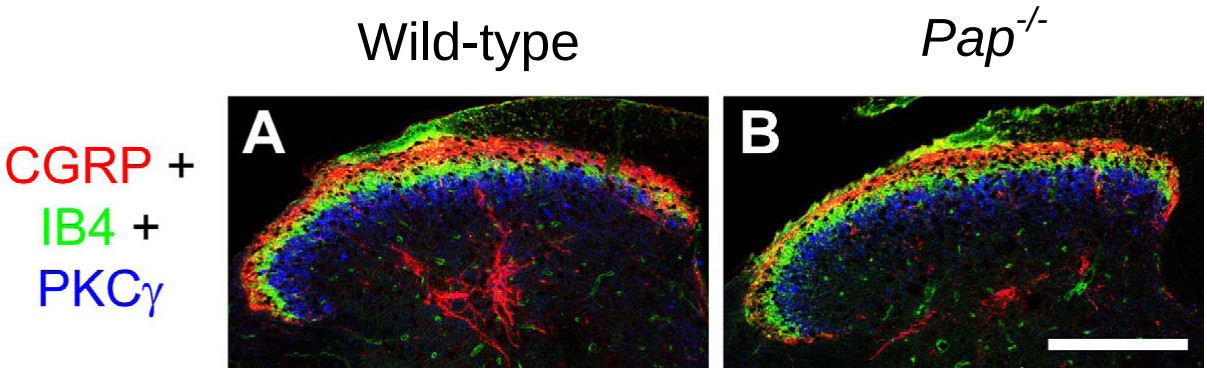

Figure 2.4. Axon terminals are anatomically normal in *Pap*$^{-/-}$ mice. Lumbar spinal cord sections from (A) wild-type and (B) *Pap*$^{-/-}$ mice were stained with antibodies to CGRP (to mark peptidergic nerve endings), isolectin B4 (IB4, to mark nonpeptidergic nerve endings) and antibodies to protein kinase C-γ (PKCγ, to mark interneurons in laminas II$_{inner}$ and III). Confocal image analysis revealed no gross anatomical differences between genotypes (n=2 mice from each genotype). Scale bar: 150 μm. (Back to text)

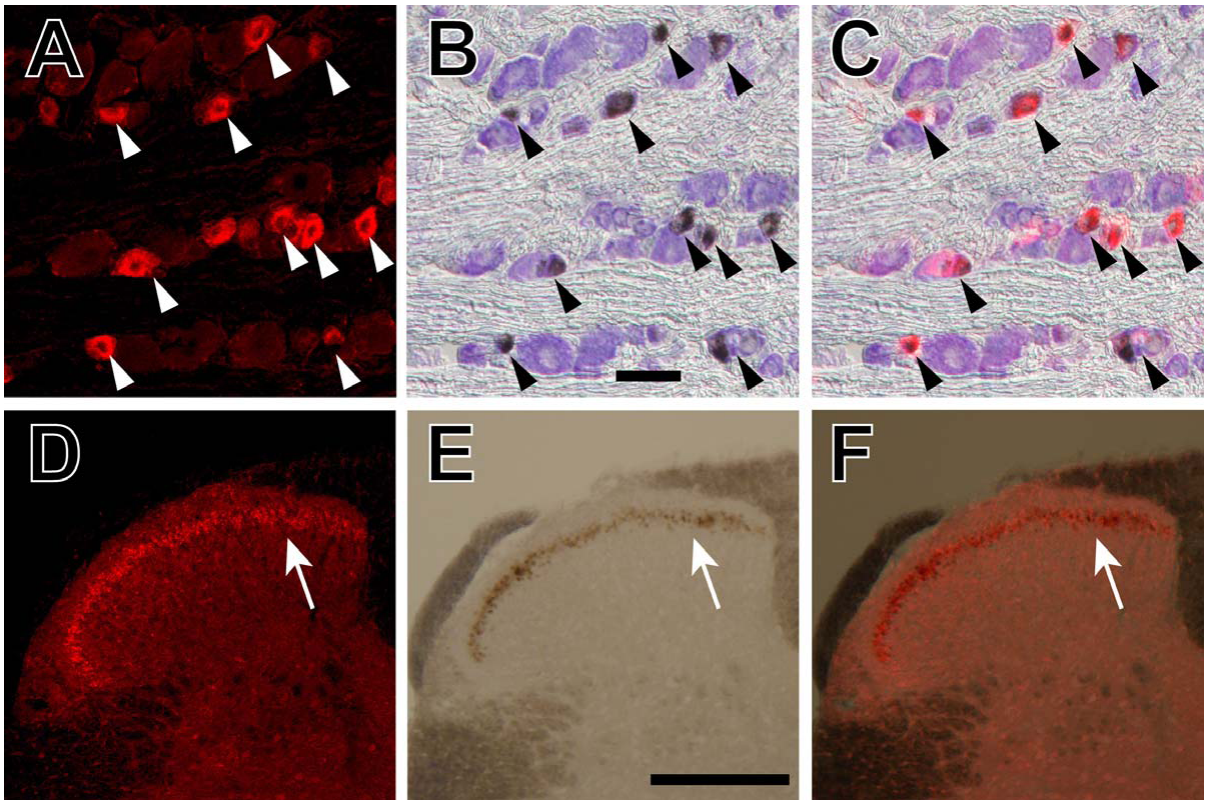

Figure 2.5. TMP histochemical activity and PAP protein are co-localized in mouse DRG neurons and on axon terminals in lamina II of the spinal cord. (A-C) Lumbar DRG and (D-F) spinal cord were immunostained with (A, D) PAP antibodies and imaged by confocal microscopy. (B) An adjacent section was then processed for TMP histochemistry and counterstained with cresyl violet. (E) The same section depicted in (D) was processed for TMP histochemistry. (C, F) Merged images. Arrowheads mark examples of double-labeled neurons. Arrow marks Lamina II. Scale bar: 50 μm in (A-C); 200 μm in (D-F). (Back to text)

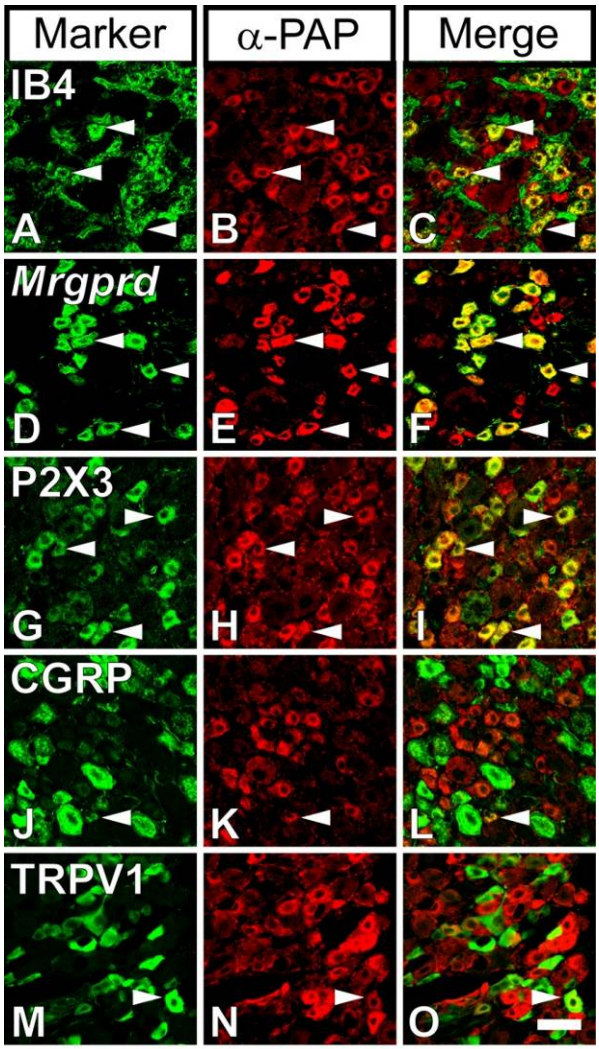

Figure 2.6. PAP is primarily expressed in nonpeptidergic neurons. (A-O) Mouse L4-L6 DRG neurons were stained with antibodies against various sensory neuron markers (green) and with antibodies against PAP (red). Tissue from adult *Mrgprd*Δ^{EGFPf} mice was used to identify *Mrgprd*-expressing neurons. Arrowheads mark examples of double-labeled cells. Images were acquired by confocal microscopy. Scale bar (bottom right panel): 50 μm for all panels. (Back to text)

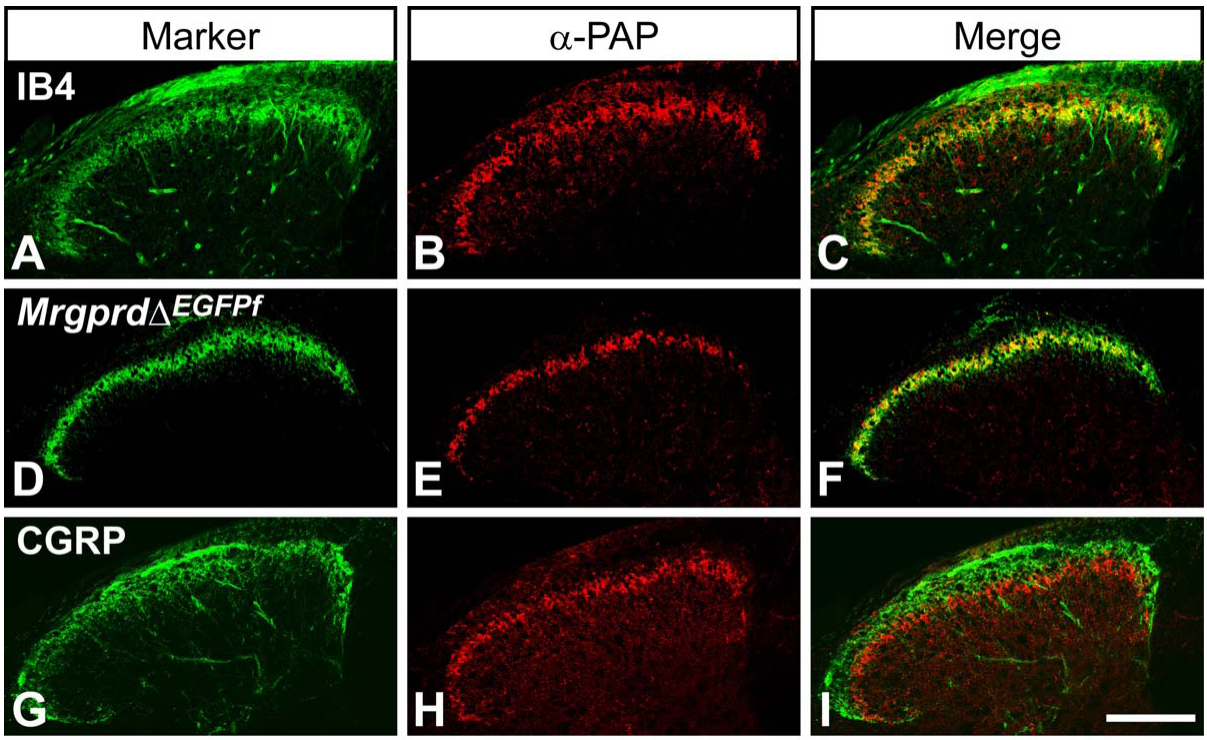

Figure 2.7. PAP protein is localized to nonpeptidergic axon terminals in lamina II of the mouse spinal cord. Lumbar spinal cord sections were double-labeled with antibodies against selected axonal markers (A, D, G; green) and PAP (B, E, H; red). IB4 and *MrgprdΔEGFPf* mark nonpeptidergic endings. CGRP marks peptidergic endings. Images were acquired by confocal microscopy. Scale bar: 150 μm for all panels. (Back to text)

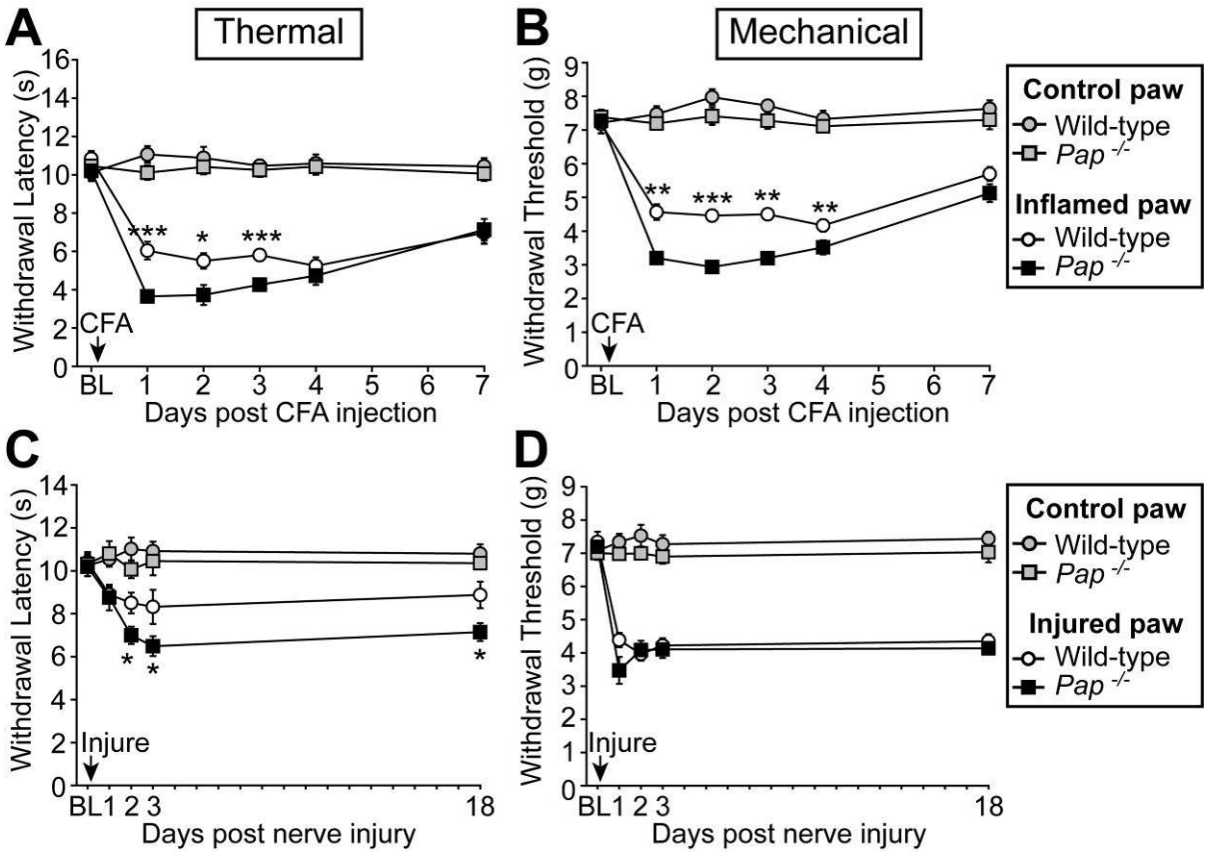

Figure 2.8 ***Pap*$^{-/-}$ mice show enhanced nociceptive responses following inflammation and nerve injury.** (A, B) CFA inflammatory pain model. Wild-type and *Pap*$^{-/-}$ mice were tested for (A) thermal sensitivity using a radiant heat source and (B) mechanical sensitivity using an electronic von Frey semi-flexible tip before (baseline, BL) and following injection of CFA (CFA-arrow) into one hindpaw. The non-inflamed hindpaw served as control. (C, D) SNI neuropathic pain model. The sural and common peroneal branches of the sciatic nerve were ligated then transected (Injure-arrow). Injured and non-injured (control) hindpaws were tested for (C) thermal and (D) mechanical sensitivity. (A-D) Paired t-tests were used to compare responses at each time point between wild-type (n=10) and *Pap*$^{-/-}$ mice (n=10); same paw comparisons. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

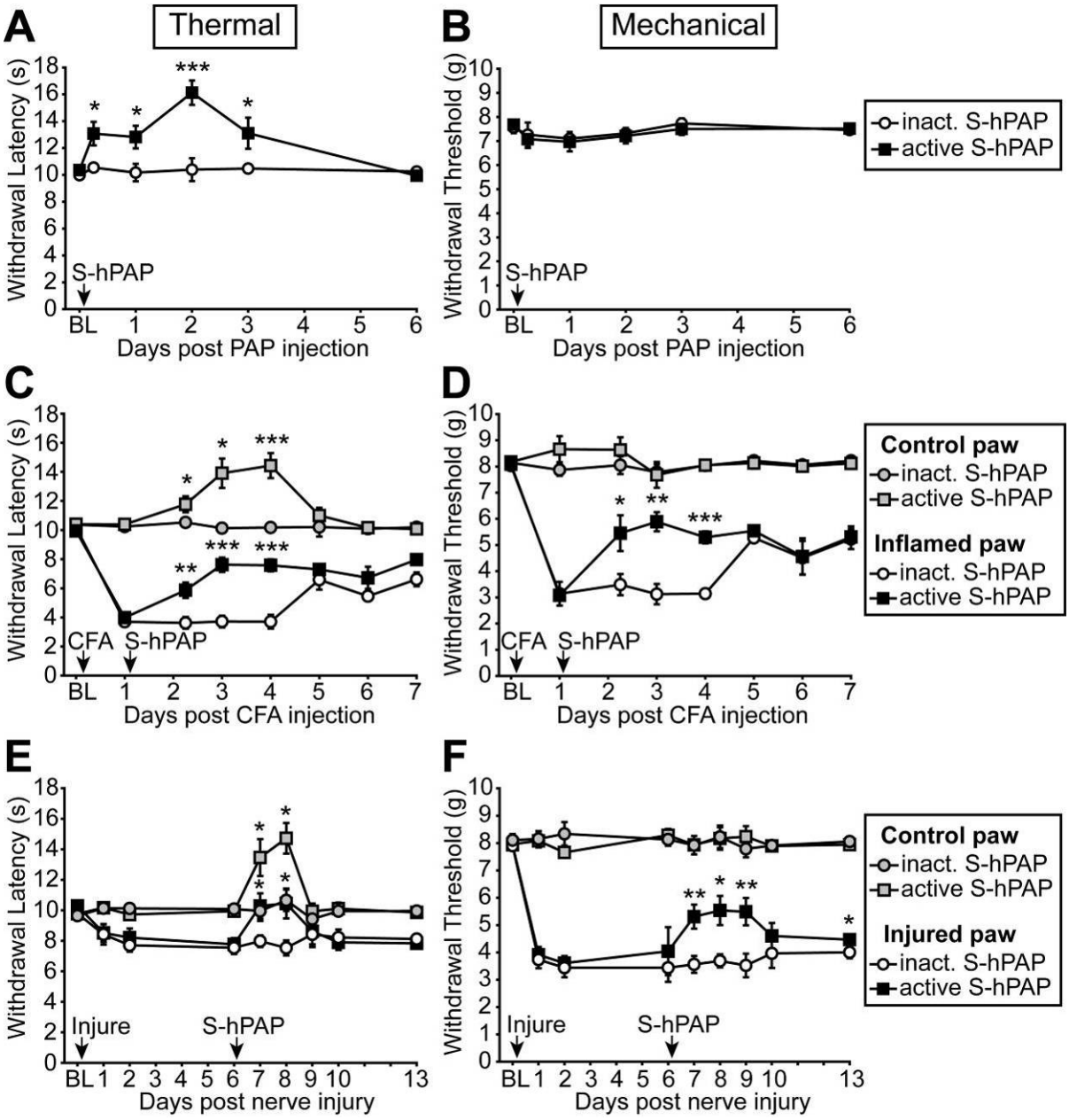

Figure 2.9. S-hPAP protein has long-lasting analgesic and antinociceptive effects when injected intraspinally. (A, B) Wild-type mice were tested for (A) thermal and (B) mechanical sensitivity before (baseline, BL) and following i.t. injection of active or inactive S-hPAP (hPAP-arrow). (C, D) CFA inflammatory pain model. CFA was injected into one hindpaw (CFA-arrow). Active or inactive S-hPAP was i.t. injected one day later (hPAP-arrow). Inflamed and non-inflamed (control) hindpaws were tested for (C) thermal and (D)

mechanical sensitivity. (E, F) SNI neuropathic pain model. The sural and common peroneal branches of the sciatic nerve were ligated then transected (Injure-arrow). Active or inactive S-hPAP was i.t. injected six days later (hPAP-arrow). Injured and non-injured (control) hindpaws were tested for (E) thermal and (F) mechanical sensitivity. (A-F) 250 mU S-hPAP injected per mouse. Paired t-tests were used to compare responses at each time point between mice injected with active hPAP (n=10 mice per experiment) to mice injected with heat-inactivated hPAP (n=10 mice per experiment); same paw comparisons. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

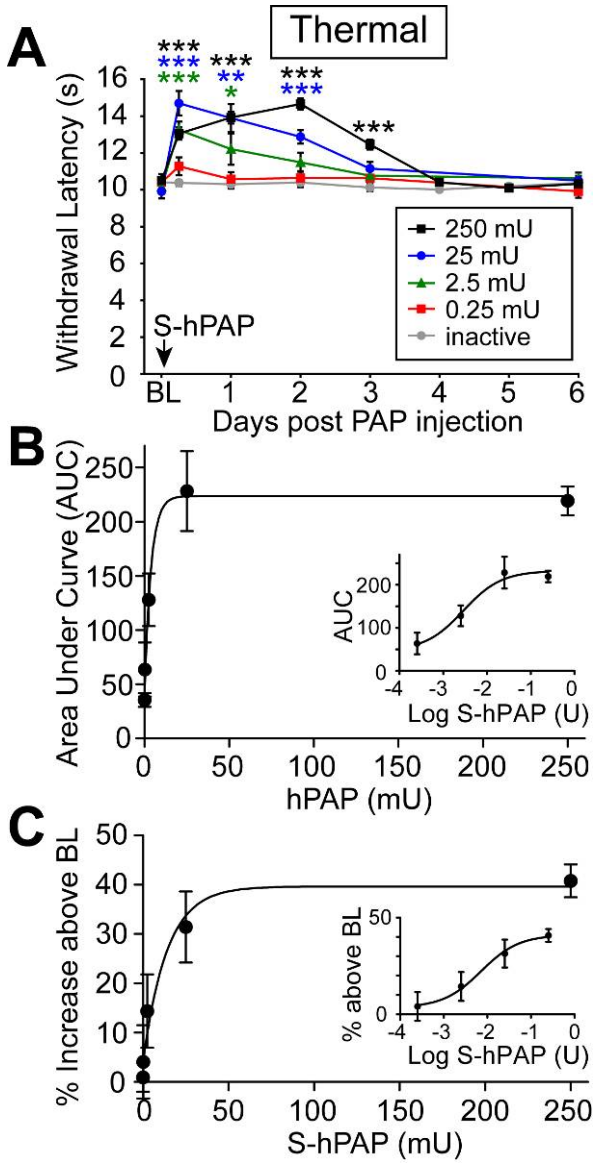

Figure 2.10. Dose-dependent anti-nociceptive effects of intrathecal S-hPAP. (A) Effects of injecting (i.t.) inactive S-hPAP or increasing amounts of active S-hPAP (hPAP-arrow) on paw withdrawal latency to the radiant heat source. (B) The same data plotted as area under the curve [AUC; units are in Latency (s) x Time post injection (h); integrated over 72 h (3 days) post injection] relative to mice injected with inactive PAP. (B-Inset) Plotted on log scale. (C) Data from the two day time points plotted as percent maximal increase in paw withdrawal latency relative to baseline (BL). (C-Inset) Plotted on log scale. (A-C) Injection

(i.t.) volume was 5 μL. n=8 wild-type mice were used for the 0.25 mU, 2.5 mU and 25 mU amounts. n=24-74 wild-type mice were used for the inactive S-hPAP and 250 mU amounts; this reflects pooled data from many of the wild-type mice used during the course of this study. All data are presented as means ± s.e.m. Curves were generated by non-linear regression analysis using Prism 5.0 (GraphPad Software, Inc). Significant differences are shown relative to baseline (paired t-tests); * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

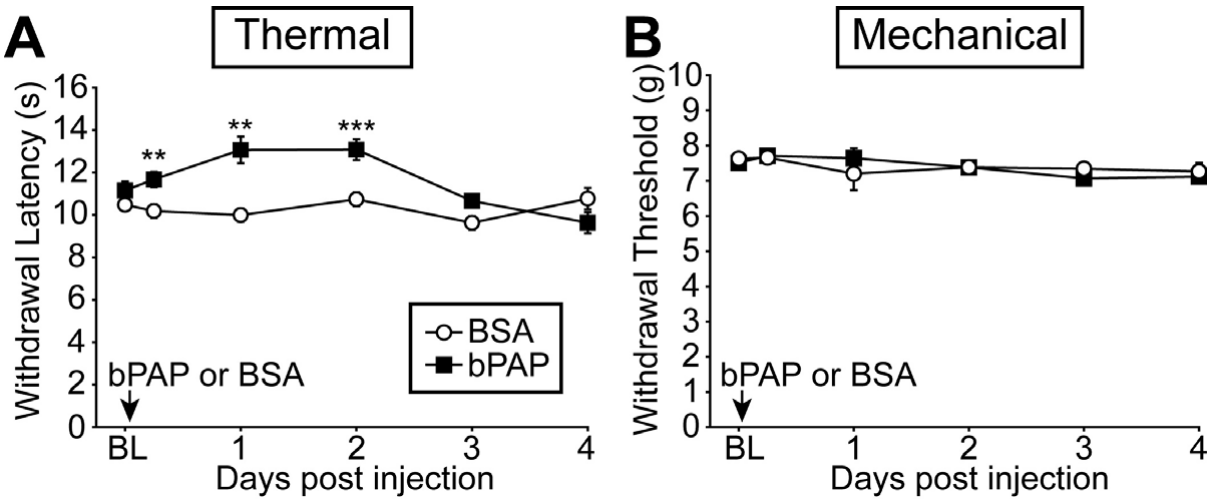

Figure 2.11. Bovine (b)PAP has anti-nociceptive effects on noxious thermal sensitivity, but not mechanical sensitivity, when injected intrathecally into lumbar spinal cord. (A, B) Wild-type mice were tested for (A) thermal and (B) mechanical sensitivity before (baseline, BL) and following i.t. injection of active bPAP (0.3 U/mL; 1.3 mg/mL) or bovine serum albumin (BSA, 1.3 mg/mL); (arrow). Paired t-tests were used to compare responses at each time point between mice injected with bPAP (n=10 mice) to mice injected with BSA (n=10 mice). Significant differences are shown; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

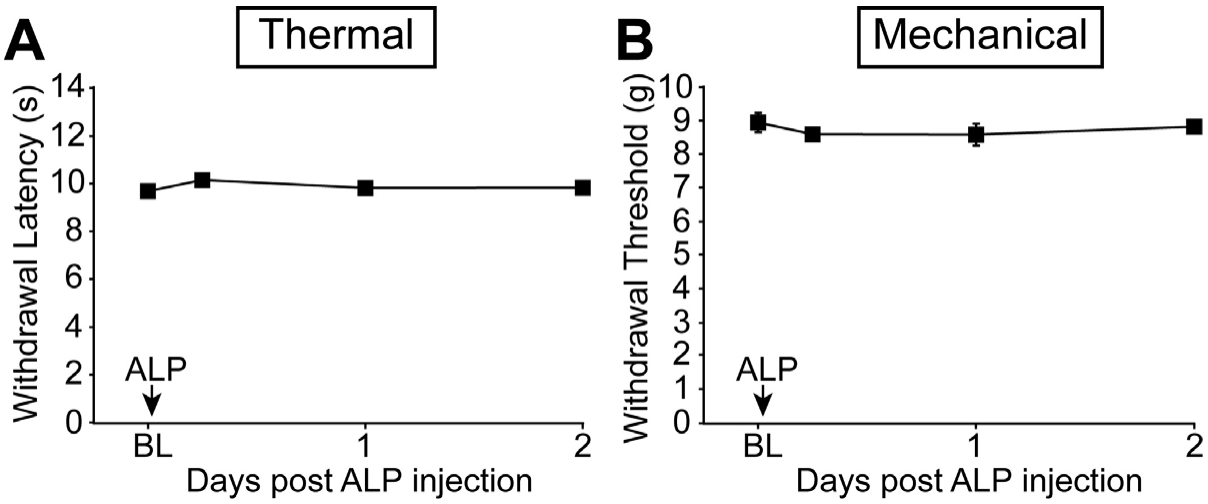

Figure 2.12. Bovine alkaline phosphatase (ALP) has no effect on noxious thermal or mechanical sensitivity when injected intrathecally into lumbar spinal cord.

Wildtype mice (n=9) were tested for (A) thermal and (B) mechanical sensitivity before (baseline, BL) and following i.t. injection of recombinant ALP (5000 U/mL; 25,000 mU total); (arrow). Note that the unit definition for PAP and ALP is essentially the same (1 U will hydrolyze 1 μmole of 4-nitrophenyl phosphate per minute at 37°C at pH 4.8 or pH 9.8, respectively). Thus, 25,000 mU ALP has 100x more phosphatase activity than the 250 mU S-hPAP we used throughout this study. Paired t-tests were used to compare responses at each time point to baseline values. There were no significant differences at any of the time points in these assays. All data are presented as means ± s.e.m. (some of the error bars are obscured due to their small size). We tested a lower concentration of ALP (250 mU, i.t.) and found that it also did not reduce thermal or mechanical sensitivity (data not shown). (Back to text)

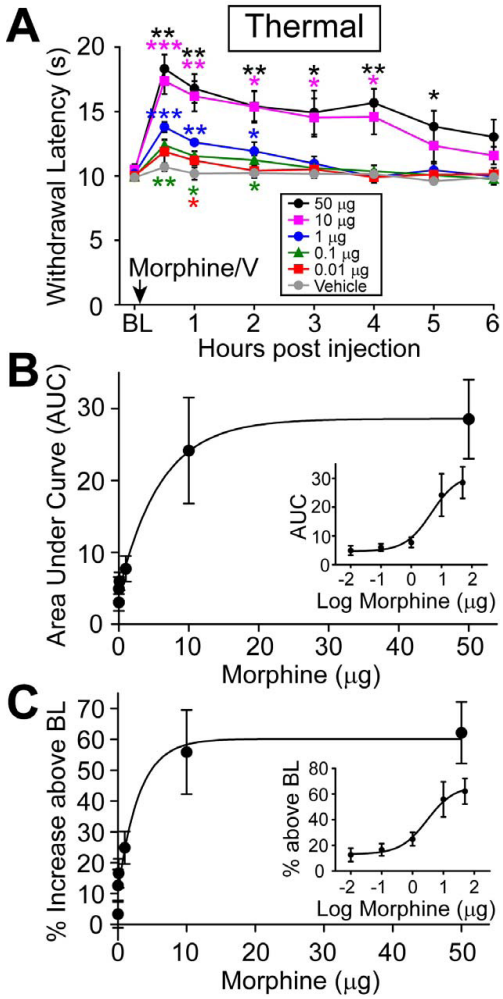

Figure 2.13. Dose-dependent anti-nociceptive effects of intrathecal morphine. Effects of injecting (i.t.) vehicle or increasing doses of morphine sulfate (Morphine/V-arrow) on paw withdrawal latency to the radiant heat source. We observed side-effects at the two highest doses [10 µg dose: three mice were paralyzed and displayed a Straub tail lasting 3-5 h. 50 µg dose: two mice died while three other mice were paralyzed and displayed a Straub tail lasting 1-2 h. Straub tail is visualized as a stiff tail held above horizontal. High doses of i.t. morphine are known to cause motor impairment and lethality (Dirig and Yaksh, 1995; Grant et al., 1995; Nishiyama et al., 2000). (B) The same data plotted as area under the curve [AUC; units are in Latency (s) x Time post injection (h); integrated over entire time

course] relative to mice injected with vehicle. (B-Inset) Plotted on log scale. (C) Data from the 1 h time points plotted as percent maximal increase in paw withdrawal latency relative to baseline (BL). (C-Inset) Plotted on log scale. (A-C) Injection (i.t.) volume was 5 μL. n=8 wild-type mice were used per dose. Curves were generated by non-linear regression analysis using Prism 5.0 (GraphPad Software, Inc). Significant differences are shown relative to baseline (paired t-tests); * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

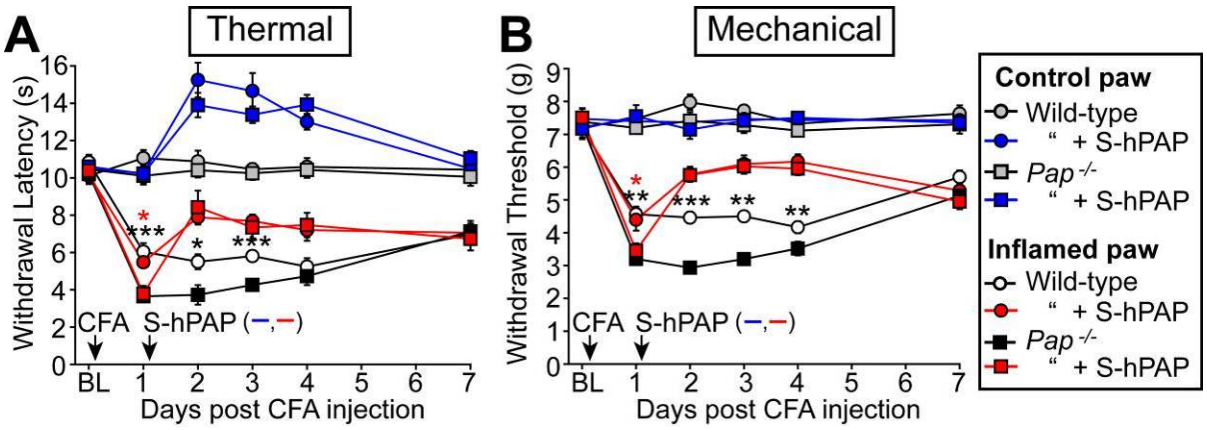

Figure 2.14. Intraspinal PAP has anti-nociceptive effects in *Pap*$^{-/-}$ mice and rescues the chronic inflammatory pain behavioral phenotype in *Pap*$^{-/-}$ mice. Wild-type and *Pap*$^{-/-}$ mice were tested for (A) thermal sensitivity and (B) mechanical sensitivity before (baseline, BL) and following injection of CFA (CFA-arrow) into one hindpaw. The noninflamed hindpaw served as control. One day later, half of the wild-type and *Pap*$^{-/-}$ mice were injected with active S-hPAP (hPAP-arrow; 250 mU, i.t.; red and blue lines) while the other half were injected with inactive S-hPAP (black lines). Note: Data from these inactive S-hPAP injected mice were presented in Figure 2.8A, B. Paired t-tests were used to compare responses at each time point between wild-type (n=10/group) and *Pap*$^{-/-}$ mice (n=10/group); same paw comparisons (n=40 mice were used for this experiment). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

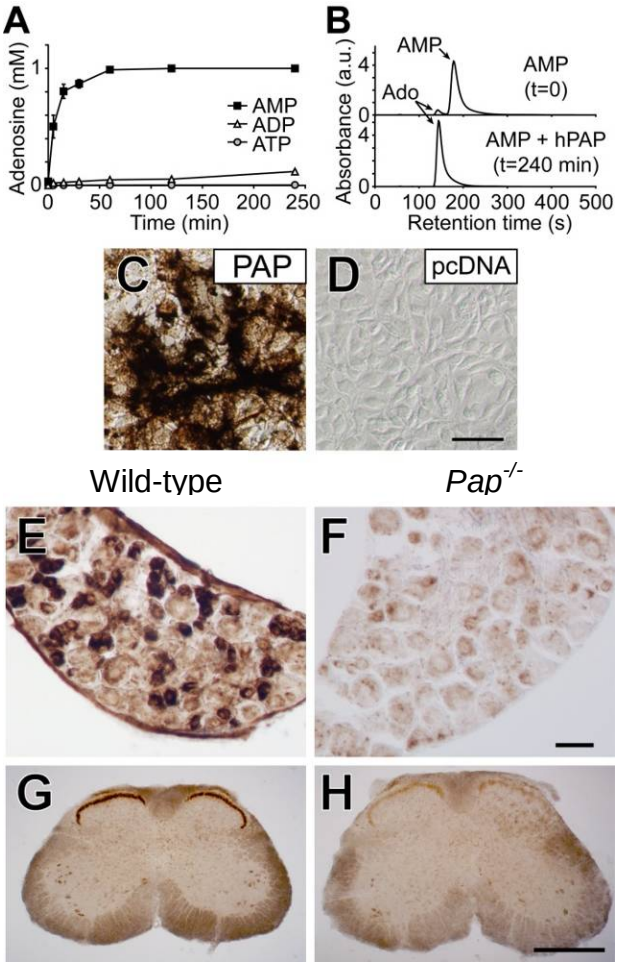

Figure 2.15. PAP has ecto-5'-nucleotidase activity as revealed by dephosphorylation of AMP to adenosine in vitro, in cells and in nociceptive circuits. **(**A) S-hPAP (2.5 U/mL) was incubated with 1 mM AMP, ADP, or ATP at pH 7.0. Reactions (n=3 per time point) were stopped by heat denaturation at the indicated times. Conversion of nucleotides to adenosine was measured by HPLC. (B) HPLC chromatogram before (t=0) and after (t=240 min) incubation of 1 mM AMP with S-hPAP. Peaks corresponding to adenosine (ado) and AMP are indicated. Arbitrary units (a.u.). (C) HEK 293 cells were transfected with a mouse TM-PAP expression construct or (D) with empty pcDNA3.1 vector and then stained using AMP histochemistry. The plasma membrane was not permeabilized, so that extracellular

phosphatase activity could be assayed. (E-F) Lumbar DRG and (G-H) spinal cord from wild-type and *Pap*$^{-/-}$ adult mice stained using AMP histochemistry. Motor neurons in the ventral horn of wild type and *Pap*$^{-/-}$ spinal cord were also stained. Identical results were obtained from five additional mice of each genotype. AMP (6 mM in (C), (D) and 0.3 mM in E-H) was used as substrate and buffer pH was 5.6. Scale bar: 5 μm in (C-F); 500 μm in (G), (H). (Back to text)

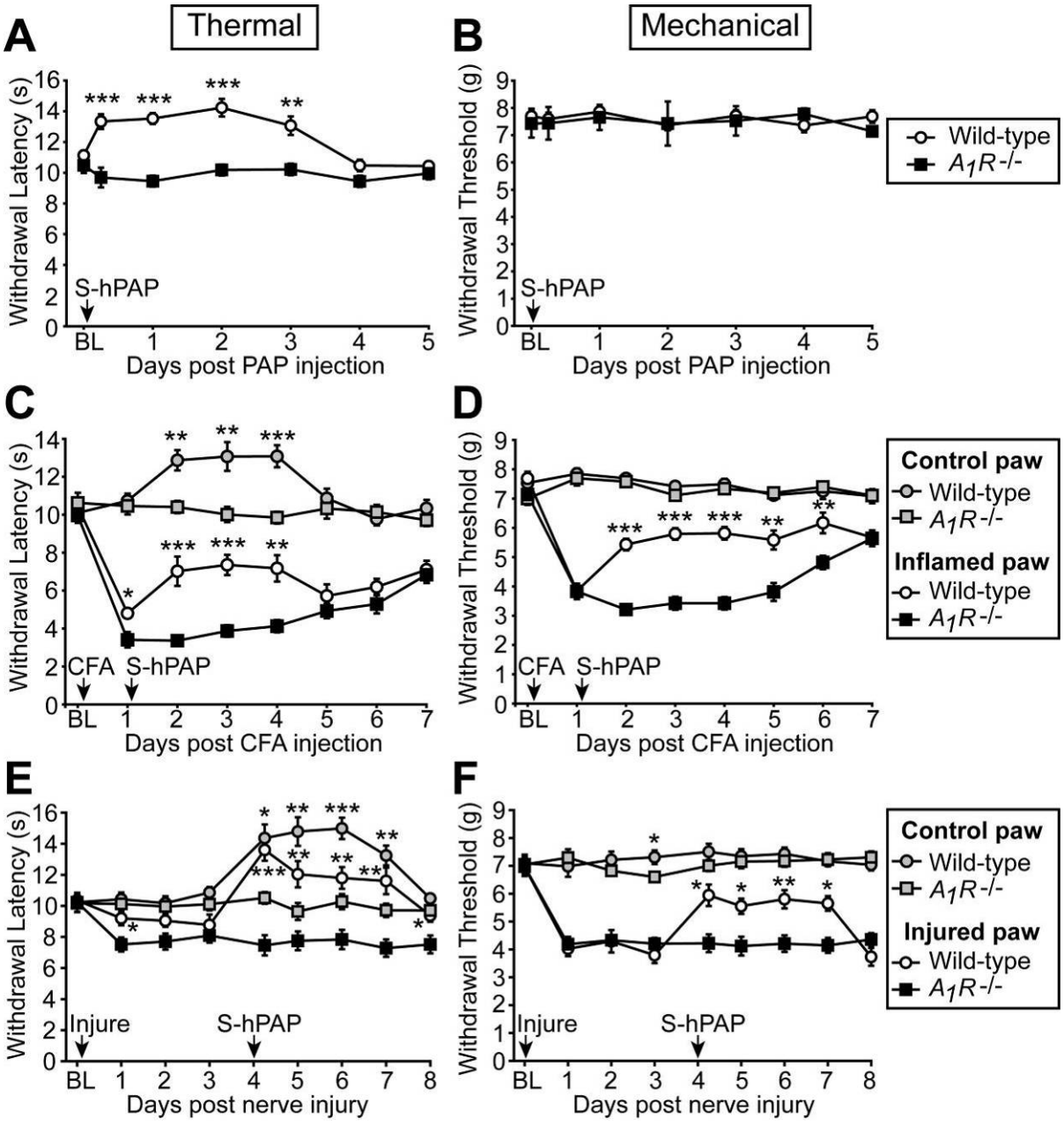

Figure 2. 16. PAP requires A_1-adenosine receptors for anti-nociception. (A, B) Wildtype and *A1R-/-* mice were tested for (A) thermal and (B) mechanical sensitivity before (baseline, BL) and following i.t. injection of S-hPAP (hPAP-arrow). (C, D) CFA was injected into one hindpaw (CFA-arrow) of wild-type and *A1R-/-* mice. Active or inactive S-hPAP was i.t. injected one day later (hPAP-arrow). Inflamed and non-inflamed (control) hindpaws were

tested for (C) thermal and (D) mechanical sensitivity. (E, F) The SNI model was used to induce neuropathic pain (Injure-arrow) in wild-type and $A_1R^{-/-}$ mice. Active or inactive S-hPAP was i.t. injected four days later (hPAP-arrow). Injured and noninjured (control) hindpaws were tested for (E) thermal and (F) mechanical sensitivity. For all experiments, 250 mU hPAP was injected per mouse. T-tests were used to compare responses at each time point between wild-type (n=10) and $A_1R^{-/-}$ mice (n=9); same paw comparisons. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

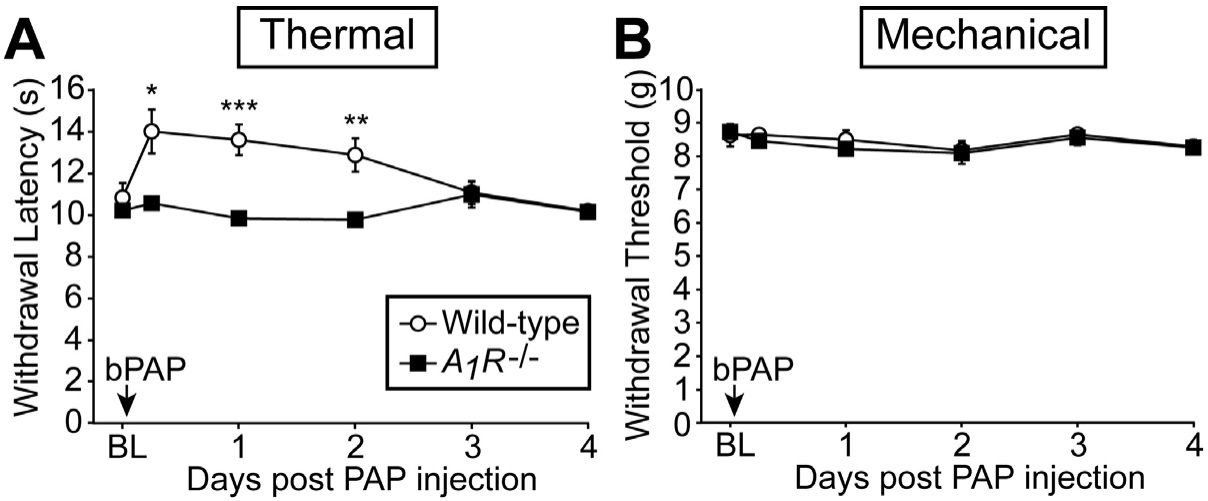

Figure 2.17. A_1-adenosine receptors are required for bovine (b)PAP anti-nociception. Wild-type mice (n=7) and *A1R-/-* mice (n=7) were tested for (A) thermal and (B) mechanical sensitivity before (baseline, BL) and following i.t. injection of active bPAP (0.3 U/mL); (arrow). Paired t-tests were used to compare responses at each time point between wild-type and knockout mice. Significant differences are shown; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

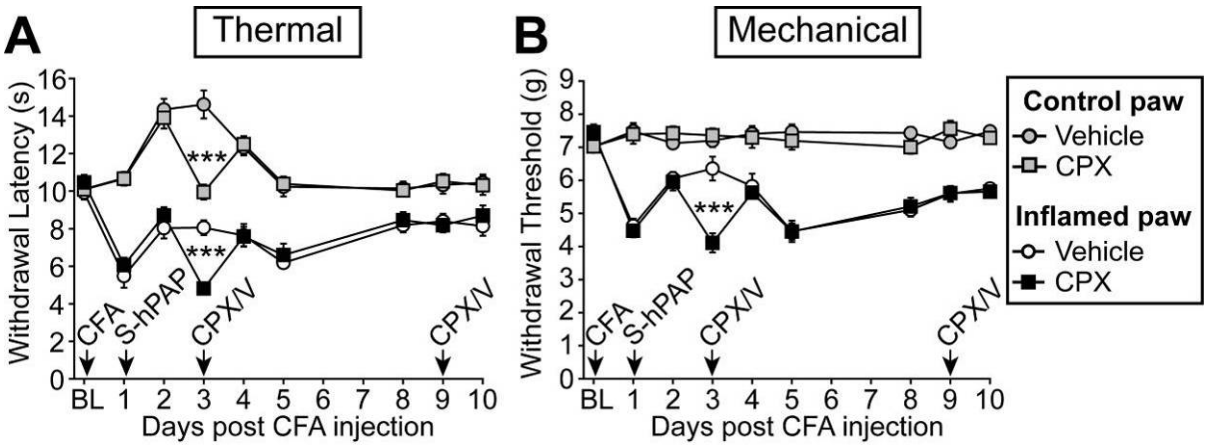

Figure 2.18. The anti-nociceptive effects of PAP can be transiently inhibited with a selective A_1R antagonist. Wild-type mice were tested for (A) noxious thermal and (B) mechanical sensitivity before (baseline, BL) and following injection of CFA (CFA arrow) into one hindpaw. The non-inflamed hindpaw served as control. All mice were injected with active S-hPAP (hPAP-arrow; 250 mU, i.t.). Two days later, half the mice were injected with vehicle (CPX/V-arrow, circles; intraperitoneal (i.p.); 1 h before behavioral measurements) while the other half were injected with 8-cyclopentyl-1, 3- dipropylxanthine (CPX/V-arrow, squares; 1 mg/kg i.p.; 1 h before behavioral measurements). CPX transiently antagonized all anti-nociceptive effects of S-hPAP. In contrast, CPX did not affect thermal or mechanical sensitivity when injected on day 9 – four days after the anti-nociceptive effects of S-hPAP wore off. These experiments highlight a selective effect of CPX on S-hPAP anti-nociception and further demonstrate that PAP suppresses pain via adenosine production and A_1R activation. Paired t-tests were used to compare responses at each time point between vehicle (n=10) and CPX-injected mice (n=10); same paw comparisons. *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

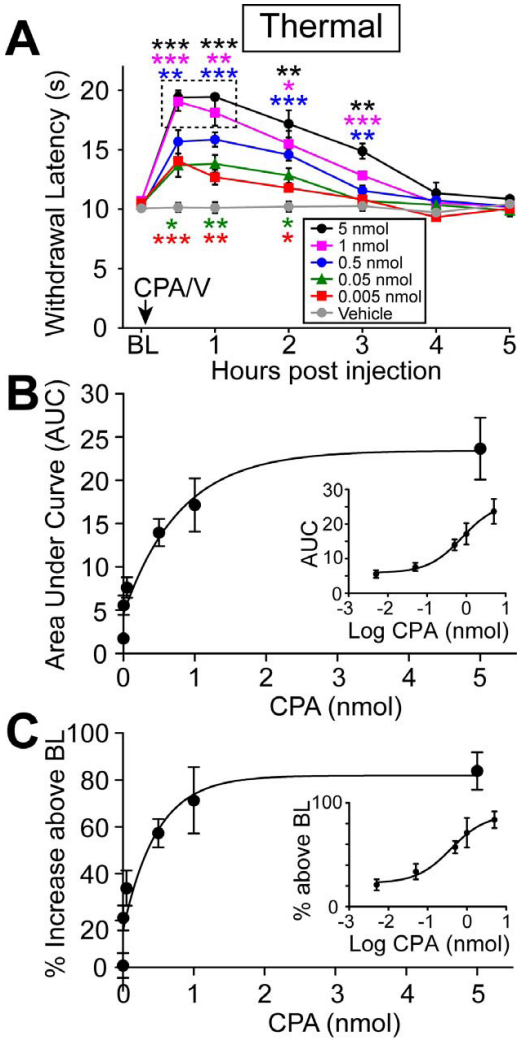

Figure 2.19. Dose-dependent anti-nociceptive effects of intrathecal N6-cyclopentyladenosine (CPA), a selective A_1-adenosine receptor agonist. Effects of injecting (i.t.) vehicle or increasing doses of CPA (CPA/V-arrow) on paw withdrawal latency to the radiant heat source. Almost all mice injected with the two highest doses of CPA reached the cutoff of 20 s because of fore- and hindlimb paralysis lasting one hour (boxed region). High doses of adenosine receptor agonists are known to cause motorparalysis (Sawynok, 2006). (B) The same data plotted as area under the curve [AUC; units are in Latency (s) x Time post injection (h); integrated over entire time course] relative to mice injected with vehicle. (B-Inset) Plotted on log scale. (C) Data from the 1 h time points plotted

as percent maximal increase in paw withdrawal latency relative to baseline (BL). (C-Inset) Plotted on log scale. (A-C) Injection (i.t.) volume was 5 μL. n=8 wild-type mice were used per dose. All data are presented as means ± s.e.m. Curves were generated by non-linear regression analysis using Prism 5.0 (GraphPad Software, Inc). Significant differences are shown relative to baseline (paired t-tests); * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

Table 2.1. Quantitative analysis of PAP and sensory neuron marker co-localization within wild-type adult mouse L4-L6 DRG neurons.

Marker	Percentage of PAP^+ neurons expressing indicated marker	Percentage of $marker^+$ neurons expressing PAP
IB4	70.6 ± 3.8	91.6 ± 2.8
Mrgprd-EGFPf	66.2 ± 3.2	99.2 ± 0.8
P2X3	84.5 ± 6.1	92.6 ± 3.1
TRPV1	19.1 ± 1.3	14.4 ± 1.3
CGRP	16.9 ± 3.9	17.1 ± 3.2

At least 350 cells were counted per combination. Data are expressed as means ± s.e.m.
(Back to text)

Table 2.2. Acute mechanical and thermal sensitivity are normal in *Pap*$^{-/-}$ mice.

Behavioral Assay	Wild-type	*Pap*$^{-/-}$
	Withdrawal threshold:	
Electronic von Frey	7.2 ± 0.4 g	7.8 ± 0.5 g
	Withdrawal latency:	
Radiant heating of hindpaw (Hargreaves Method)	9.1 ± 0.7 s	9.9 ± 0.9 s
Tail immersion at 46.5°C	18.4 ± 2.8 s	16.4 ± 1.6 s
Tail immersion at 49.0°C	9.9 ± 0.7 s	9.8 ± 0.9 s
Hot plate at 52°C	20.0 ± 1.1 s	19.3 ± 1.3 s

Data are expressed as means ± s.e.m. No significant differences between genotypes in any of the listed behavioral assays, paired t-test, P>0.05. n=10 male mice tested per genotype for all assays except hotplate and tail immersion at 49°C. For these latter two assays, n=14 mice (8 females, 6 males) were tested per genotype. (Back to text)

CHAPTER 3

Recombinant Mouse PAP has pH-Dependent Ectonucleotidase Activity and Acts through A_1-adenosine Receptors to Mediate Antinociception

ABSTRACT: Prostatic acid phosphatase (PAP) is expressed in nociceptive neurons and functions as an ectonucleotidase. When injected intraspinally, the secretory isoform of human and bovine PAP protein have potent and long-lasting antinociceptive effects that are dependent on A_1-adenosine receptor (A_1R) activation. In this study, we purified the secretory isoform of mouse (m)PAP using the baculovirus expression system to determine if recombinant mPAP also had antinociceptive properties. We found that mPAP dephosphorylated AMP, and to a much lesser extent, ADP at neutral pH (pH 7.0). In contrast, mPAP dephosphorylated all purine nucleotides (AMP, ADP, ATP) at an acidic pH (pH 5.6). The transmembrane isoform of mPAP had similar pH-dependent ectonucleotidase activity. A single intraspinal injection of mPAP protein had long-lasting (three day) antinociceptive properties, including antihyperalgesic and antiallodynic effects in the Complete Freund's Adjuvant (CFA) inflammatory pain model. These antinociceptive effects were transiently blocked by the A_1R antagonist 8-cyclopentyl-1, 3-dipropylxanthine (CPX), suggesting mPAP dephosphorylates nucleotides to adenosine to mediate antinociception just like human and bovine PAP. These studies indicate that PAP has species-conserved antinociceptive effects and has pH-dependent ectonucleotidase activity. The ability to metabolize nucleotides in a pH-dependent manner could be relevant to conditions like

inflammation where tissue acidosis and nucleotide release occur. Lastly, our studies demonstrate that recombinant PAP protein can be used to treat chronic pain in animal models.

This work was previously published: Sowa, N.A., Vadakkan, K.I., and Zylka, M.J. (2009). *PLoS ONE* 4, e4248.

3.1) Introduction

Small-diameter dorsal root ganglia (DRG) neurons contain a classic, histochemically-defined enzyme known as Fluoride-Resistant Acid Phosphatase (FRAP) or Thiamine Monophosphatase (TMPase) (Csillik and Knyihar-Csillik, 1986; Dodd et al., 1983). Recently, we found that TMPase was molecularly equivalent to Prostatic Acid Phosphatase (PAP, also known as ACPP) (Zylka et al., 2008). In mammals, PAP is expressed as a secreted protein or as a transmembrane protein (Quintero et al., 2007; Roiko et al., 1990; Vihko, 1979). These isoforms have identical N-terminal regions, including a signal peptide and extracellular acid phosphatase domain, but differ at the C-terminus due to the inclusion or exclusion of a transmembrane domain. Using in situ hybridization with isoform-specific riboprobes, we found that small-diameter DRG neurons primarily express the transmembrane isoform of PAP (Zylka et al., 2008). Moreover, using immunohistochemistry, we found that PAP protein is localized to a majority of all nonpeptidergic nociceptive neurons, a subset of peptidergic nociceptive neurons and to axon terminals located in lamina II of the dorsal spinal cord (Zylka et al., 2008).

We also found that PAP functions in nociceptive circuits as an ectonucleotidase by dephosphorylating adenosine monophosphate (AMP) to adenosine (Zylka et al., 2008). This was based on our observation that intrathecal injection of human (S-h)PAP protein (the secreted isoform) produced long-lasting antinociceptive, antihyperalgesic and antiallodynic effects that were dependent on A_1-adenosine receptor (A_1R) activation (Zylka et al., 2008). These antinociceptive effects were eight-times more effective than the commonly used analgesic morphine. When injected intrathecally, bovine (b)PAP also had long-lasting antinociceptive effects that were dependent on A_1R activation. Conversely, PAP knockout

(*Pap*$^{-/-}$) mice showed enhanced sensitivity in animal models of chronic inflammatory pain and neuropathic pain (Zylka et al., 2008), a phenotype that was similar to $A_1R^{-/-}$ mice (Wu et al., 2005). Lastly, dephosphorylation of extracellular AMP was greatly reduced in small-diameter DRG neurons and dorsal spinal cord of *Pap*$^{-/-}$ mice.

For our initial study, we used secretory isoforms of PAP that were purified from human seminal fluid and from bovine prostate (Zylka et al., 2008). The secretory isoforms of human, bovine and mouse PAP are ~80% identical to one another at the amino acid level (based on pairwise sequence comparisons), suggesting they might have similar antinociceptive effects in vivo. At the time we performed our initial studies, we were unable to test mPAP protein for antinociceptive effects because there were no commercially available sources of pure mPAP protein. Moreover, without pure protein, we could not determine the substrate specificity for secretory mPAP. To overcome these limitations, we synthesized and purified recombinant mPAP protein (secretory isoform). Strategies for generating recombinant human and rat PAP protein were previously described (Ostanin et al., 1994; Vihko et al., 1993). At neutral pH, mPAP primarily dephosphorylated AMP. In addition, we found that mPAP could dephosphorylate all purine nucleotides (AMP, ADP, ATP) under acidic pH conditions. This suggested a broader function for PAP in nucleotide metabolism and has implications in inflammatory pain conditions where extracellular pH is reduced.

Recombinant proteins can be produced in large quantities, purified for use in humans (Burch et al., 2000; Dorr, 1993; Zucchini, 2008) and are not likely to be contaminated with human pathogens. Thus, the approaches outlined in this study could be used to purify and test recombinant mouse or human PAP as a treatment for chronic pain in humans.

3.2) Materials and Methods

3.2.1) Molecular Biology and Protein Purification

The mPAP-Tr-$(His)_6$ baculovirus expression clone (encompassing nt 61-1206 from GenBank accession # NM_019807) was generated by PCR amplification, using a full-length expression construct of mPAP (secreted isoform) as template and Phusion polymerase. PCR products were cloned into pFastBac1 (Invitrogen) and completely sequenced. Primer sequences contained XbaI sites (underlined) to facilitate cloning (N-terminal primer: 5'-cgctctagaaccatgcgagccgttcctctgc. C-terminal thrombin-$(His)_6$ tag primer: 5'-gcgtctagattaatgatgatgatgatgggagccacgcggaaccagattccgtccttggtggctgc). There are no thrombin cleavage sites in the mPAP protein except for the cleavage site we introduced. This vector was then used to generate recombinant mPAP protein using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Briefly, we infected Hi5 insect cells with high-titer recombinant baculovirus, incubated the cells for 48 hours at 27°C, then harvested and concentrated the supernatant containing secreted mPAP protein. Then, mPAP protein was purified from the concentrated supernatant using Ni-NTA HisTrap agarose (GE Healthcare Life Sciences) affinity chromatography and imidazole as eluant. Lastly, mPAP protein was dialyzed against PBS to remove imidazole. Protein purity was confirmed by SDS-PAGE, staining for total protein with GelCode Blue (Pierce/Thermo Scientific, Cat. # 24590) and western blotting with Penta-His antibody (Qiagen, Cat. # 34660). Amersham full-range rainbow molecular weight markers (GE Healthcare) were used for SDS-PAGE and MagicMark XP markers (Invitrogen, Cat. # LC5602) were used for western blots. This purification strategy is based on the observation that recombinant rat PAP is secreted into the

medium of baculovirus-infected insect cells (Vihko et al., 1993). Recombinant mPAP was kept at 4°C for short-term (1-2 months) use and at -80°C for long-term storage.

3.2.2) Enzyme Assays

Enzymatic reactions (50 μL final) were carried out with recombinant mPAP at 37°C for 3 minutes in 10 mM sodium acetate, pH 5.6 or 10 mM HEPES, pH 7.0 with AMP, ADP or ATP as substrate. Reactions were stopped by adding 950 μL of the malachite green color reagent [0.03% (w/v) malachite green oxalate, 0.2% (w/v) sodium molybdate, 0.05% (v/v) Triton X-100, dissolved in 0.7 M HCl] then incubating at room temperature for 30 minutes. Inorganic phosphate was quantified by measuring OD_{650} and comparing to an inorganic phosphate (KH_2PO_4) standard curve (Lanzetta et al., 1979).

Enzyme activity of mPAP was determined using 4-nitrophenyl phosphate as substrate following Sigma's Quality Control Test Procedure for PAP (SSPNPP11, revision 8/29/97). Unit (U) definition: 1 U hydrolyzes 1 μmole of 4-nitrophenyl phosphate per minute at 37°C at pH 4.8.

3.2.3) Cell Culture and Histochemistry

HEK 293 cells were cultured and transfected as previously described (Zylka et al., 2008). Enzyme histochemistry was performed as previously described (Zylka et al., 2008) using 6 mM AMP, ADP, or ATP as substrate and Tris-maleate buffer at pH 5.6 or 7.0.

3.2.4) Behavior

All behavioral experiments involving vertebrate animals were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

C57BL/6 male mice, 2-4 months old, were purchased from Jackson Laboratories and used for all behavioral experiments. All mice were acclimated to the testing room, equipment and experimenter for at least three days before behavioral testing. The experimenter was blind to drug treatment during behavioral testing.

Thermal sensitivity was measured by heating one hindpaw with a Plantar Test apparatus (IITC) following the Hargreaves method (Hargreaves et al., 1988). The radiant heat source intensity (Plantar test apparatus, IITC) was calibrated so that a paw withdrawal reflex was evoked in ~10 s., on average, in wild-type C57BL/6 mice. Cutoff time was 20 s. One measurement was taken from each paw per time point to determine paw withdrawal latency. Mechanical sensitivity was measured using semi-flexible tips attached to an Electronic von Frey apparatus (IITC) as described elsewhere (Cunha et al., 2004; Inoue et al., 2004). Three measurements were taken from each paw (separated at 10 min intervals) then averaged to determine paw withdrawal threshold in grams. To induce inflammatory pain, 20 μL Complete Freunds Adjuvant (CFA, from MP Biomedicals) was injected into one hindpaw, centrally beneath glabrous skin, with a 30G needle. 8-cyclopentyl-1, 3-dipropylxanthine (C101, Sigma) was dissolved in 0.9% saline containing 5% DMSO, 1.25% 1 M NaOH for i.p. injection. Sedation and motor dysfunction were assessed by visually observing motor activity following injections. None of the mPAP-injected mice displayed reduced mobility or paralysis following injection.

3.2.5) Intrathecal Injections

We used concentrated mPAP protein (in PBS; 1.1 mg/mL; 400 U/mL) or diluted mPAP (in 0.9% saline) for injections. mPAP was heat-inactivated by incubating at 65ºC for 40 min. Loss of activity was confirmed using the EnzChek Phosphatase Assay Kit (Invitrogen, E12020) following the manufacturer's protocol. Active or heat-inactivated mPAP was intrathecally injected (5 μL) into unanesthetized mice using the direct lumbar puncture method (Fairbanks, 2003).

3.3) Results

3.3.1) Purification of Recombinant mPAP using the Baculovirus Expression System

Large quantities of recombinant human or rat PAP (secretory isoform) can be generated in yeast or baculovirus expression systems (Ostanin et al., 1994; Vihko et al., 1993). We generated a baculovirus expression construct containing the entire open-reading frame of secretory mPAP, encompassing the signal peptide (SP) and catalytic domain fused to a C-terminal thrombin-hexahistidine (Tr-H_6) epitope tag (Figure 3.1A, 3.1B). Although the thrombin cleavage site can be used to efficiently remove the epitope tag (Figure 3.1B, data not shown), we performed our studies below with recombinant mPAP-Tr-H_6 (henceforth referred to as mPAP) containing the C-terminal epitope tag because removal of the tag required additional purification steps and did not impact enzyme activity.

We detected large quantities of mPAP protein in the tissue culture supernatant of Hi5 insect cells two days after infection with recombinant baculovirus. We purified mPAP from the supernatant in one step, using nickel chelate affinity chromatography. We confirmed protein purity by running mPAP on an SDS-PAGE gel and staining for total protein (Figure

3.1C) and western blotting (Figure 3.1D). In both cases, we observed one predominant band at ~45 kDa, corresponding to the calculated molecular weight of monomeric mPAP (45.2 kDa). The weakly stained ~90 kDa band on our overloaded western blot likely reflects a small amount of non-denatured mPAP, consistent with the fact that native PAP is a dimer (Ostrowski and Kuciel, 1994; Schneider et al., 1993). No additional bands were observed, indicating that mPAP protein was pure and largely intact. This purified, recombinant mPAP protein effectively dephosphorylated the generic acid phosphatase substrates para-nitrophenyl phosphate (p-NPP) and 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) and was inhibited by the acid phosphatase inhibitor L-(+)-tartrate (IC_{50}=1.45 mM; Figure 3.2). Recombinant human and rat PAP are similarly inhibited by L-(+)-tartrate (Ostanin et al., 1994; Porvari et al., 1994; Vihko et al., 1993).

3.3.2) Recombinant mPAP Dephosphorylates Purine Nucleotides in a pH-dependent Manner

We previously found that S-hPAP (secreted isoform) generated adenosine by dephosphorylating AMP and, to a much lesser extent, ADP at neutral pH (Zylka et al., 2008). To determine if secretory mPAP had similar substrate specificity and to evaluate pH dependence, we incubated mPAP with AMP, ADP or ATP at pH 7.0 or pH 5.6, then detected inorganic phosphate using the malachite green assay. We found that mPAP dephosphorylated AMP and, to a lesser extent, ADP at neutral pH (Figure 3.3A), consistent with our previous findings using hPAP (Zylka et al., 2008). At pH 5.6, mPAP dephosphorylated AMP and ADP, and to a lesser extent, ATP (Figure 3.3B). This latter finding was consistent with a previous study showing that secretory hPAP could

dephosphorylate all nucleotides under acidic conditions with a rank order AMP > ADP > ATP (Vihko, 1978).

We previously found that the transmembrane isoform of mouse PAP (TM-PAP) could dephosphorylate extracellular AMP at pH 5.6 using enzyme histochemistry, indicating that PAP had ectonucleotidase activity (Zylka et al., 2008). At the time, we did not test hydrolysis at neutral pH or hydrolysis of other nucleotides. To determine if TM-PAP could dephosphorylate additional nucleotides extracellularly, and if dephosphorylation was pH dependent, we transfected mouse TM-PAP into HEK 293 cells and stained non-permeabilized cells using enzyme histochemistry. Use of non-permeabilized cells allowed us to measure extracellular nucleotide hydrolysis in a cellular context. At pH 7.0, TM-PAP transfected cells were heavily stained using AMP as substrate and much less intensely stained using ADP as substrate (Figure 3.4A-C). At pH 5.6, TM-PAP transfected cells were heavily stained using AMP and moderately stained using ADP as substrate (Figure 3.4D-F). Control cells transfected with the fluorescent protein Venus were not intensely stained under any of the conditions examined (Figure 3.4G-L). When taken together, these data suggest TM-PAP has pH-dependent ectonucleotidase activity, with AMP being the preferred substrate at neutral pH and AMP and ADP being substrates at acidic pH. Moreover, these data suggest PAP could generate adenosine following hydrolysis of AMP, ADP or ATP under acidic pH conditions (Zylka et al., 2008).

3.3.3) Recombinant mPAP has Long-lasting Antinociceptive Properties

A single intrathecal injection of S-hPAP protein has antinociceptive, antihyperalgesic and antiallodynic effects that last for three days (Zylka et al., 2008). To determine if mPAP

also had long-lasting antinociceptive effects, we intrathecally (i.t.) injected wild-type mice with two doses of recombinant mPAP protein (Figure 3.5). Control mice were injected i.t. with heat-denatured, and hence phosphatase-inactive, mPAP. We then measured noxious thermal and mechanical sensitivity before (baseline, BL) and after mPAP injections. Six hours after i.t. injection, paw withdrawal latency to the noxious thermal stimulus was significantly increased relative to controls and remained elevated for three days (Figure 3.5A). This antinociceptive effect was dose-dependent and required catalytic activity, as evidenced by loss of antinociception upon heat-inactivation of mPAP (Figure 3.5A). Active mPAP did not alter mechanical sensitivity (Figure 3.5B) nor did it cause paralysis or sedation.

We next tested mPAP for antihyperalgesic and antiallodynic effects using the CFA inflammatory pain model. To do this, we injected CFA into one hindpaw to induce inflammation and used the non-inflamed paw as a control. Intrathecal injection of mPAP produced a significant increase in withdrawal latency to the noxious thermal stimulus (relative to latency on Day 1, pre-injection) in the inflamed paw (Figure 3.6A, white open circles). This antihyperalgesic effect persisted for three days. mPAP also caused a significant increase in paw withdrawal latency in the non-inflamed paw (Figure 3.6A, grey open circles, relative to day 1 values), reproducing results presented in Figure 3.5A. In addition, mPAP produced a significant increase in withdrawal threshold to the mechanical stimulus (relative to latency on Day 1, pre-injection) only in the inflamed paw (Figure 3.6B, white open circles). This antiallodynic effect lasted for three days.

3.3.4) mPAP acts through A_1Rs to Mediate Antinociception

The antinociceptive, antihyperalgesic and antiallodynic effects of S-hPAP are dependent on A_1R activation (Zylka et al., 2008). Since our biochemical experiments suggested that mPAP could generate adenosine by dephosphorylating nucleotides, we next evaluated whether mPAP had antinociceptive properties that were dependent on A_1R activation. To do this, we injected a second group of CFA-inflamed mice with the selective A_1R antagonist 8-cyclopentyl-1, 3-dipropylxanthine (CPX; 1 mg/kg i.p.). CPX transiently antagonized all antinociceptive effects of mPAP, including the antihyperalgesic (Figure 3.6A) and antiallodynic (Figure 3.6B) effects. This same i.p. dose of CPX did not affect thermal or mechanical sensitivity in the control or CFA-inflamed paw once the antinociceptive effects of PAP wore off (see Figure S10 in (Zylka et al., 2008)). Taken together, these data suggest that the antinociceptive effects of mPAP were due to ectonucleotidase-dependent generation of adenosine followed by activation of A_1Rs.

3.4) Discussion

We previously found that PAP was expressed in nociceptive neurons and functioned as an ectonucleotidase by dephosphorylating AMP to adenosine. Moreover, PAP had antinociceptive properties that were dependent on A_1R activation (Zylka et al., 2008). At the time, we could not perform in vivo gain-of-function studies with mPAP because there were no commercially available sources of secretory mPAP protein. To overcome this limitation, we generated and purified recombinant mPAP protein and then studied the biochemical properties of mPAP and the effects of mPAP on pain sensitivity.

Our studies revealed that recombinant mPAP has very similar biochemical properties when compared to PAP from other mammalian species, including human (Ostrowski and

Kuciel, 1994; Vihko et al., 1993). Both mPAP and hPAP are inhibited by L-(+)-tartrate (Figure 3.2), both predominantly dephosphorylate AMP at neutral pH (Figure 3.3A; (Zylka et al., 2008)) and both dephosphorylate all adenine nucleotides (with relative activity AMP>ADP>ATP) at acidic pH (Figure 3.3B, (Vihko, 1978)). The Km values (0.9 – 1.6 mM) we obtained for mPAP using AMP as substrate were within the range of Km values (0.37 – 2 mM) reported for hPAP using AMP as substrate (Dziembor-Gryszkiewicz et al., 1978; Dziembor and Ostrowski, 1971; Lam et al., 1973). Mouse TM-PAP also dephosphorylated extracellular adenine nucleotides in a pH-dependent manner, although ATP was not a substrate for TM-PAP as it was for secretory PAP. This substrate discrepancy could reflect biochemical differences between these isoforms. Or, more likely, this reflects reduced sensitivity of the histochemical assay relative to the in vitro enzyme assay. When taken together, our findings suggest PAP functions as an ecto-5' nucleotidase (with relative selectivity for AMP) at neutral pH and as a generic ectonucleotidase (with selectivity for AMP, ADP and ATP) at acidic pH.

This pH-dependent hydrolysis of purine nucleotides is intriguing, especially when considering that tissue injury produces an "inflammatory soup" containing protons and nucleotides (Julius and Basbaum, 2001). Protons produce tissue acidosis, modulate the capsaicin receptor TRPV1 and activate acid-sensing ion channels (ASICs) half-maximally at pH values ranging from 4.9 to 6.8 (Caterina et al., 1997; Waldmann et al., 1997; Wemmie et al., 2008). ATP and ADP activate purinergic P2X and P2Y receptors (Burnstock, 2007; Stucky et al., 2004). Stimulation of these diverse receptors sensitizes nociceptive neurons, activates spinal microglia and causes pain (Burnstock, 2007; Nakagawa et al., 2007; Sawynok, 2007; Tozaki-Saitoh et al., 2008; Tsuda et al., 2005). PAP is extensively co-

localized with the ATP receptor P2X3 and is co-localized in 14.4% of all $TRPV1^{+}$ DRG neurons in the mouse (Zylka et al., 2008). Since PAP protein is localized on peripheral terminals of these neurons (Zylka et al., 2008) and can dephosphorylate adenine nucleotides at acidic pH, PAP could metabolize pain-producing ATP and ADP in the inflammatory soup and reduce the subsequent sensitization of nociceptive neurons. This is consistent with our observation that *Pap*$^{-/-}$ mice show enhanced thermal hyperalgesia and mechanical allodynia following inflammation (Zylka et al., 2008).

In addition, PAP is localized on the central terminals of nociceptive neurons (Zylka et al., 2008) and could metabolize nucleotides to adenosine in a pH-dependent manner at central synapses. The pH of synaptic vesicles is 5.6 ± 0.7 (Miesenbock et al., 1998) and intense neural activity can lead to acidosis within synapses that lasts for seconds (Wemmie et al., 2008). Likewise, inflammation, tissue injury and repetitive stimulation cause acidosis of up to 0.25 pH units in the dorsal horn of spinal cord when measured with pH-sensitive microelectrodes (Chesler and Kaila, 1992; Sykova and Svoboda, 1990; Sykova et al., 1988). Considering the size of these microelectrodes relative to the small volume of a synapse, these microelectrode recordings likely underestimate the magnitude of the pH change that occurs within the confines of a synapse. Thus, PAP may be exposed to low extracellular or endosomal pH when spinal synapses are activated for sustained periods of time.

Intrathecal injection of mPAP produced dose-dependent, potent and long-lasting (3 days) antihyperalgesic effects that were specific for the thermal modality in uninjured animals (Figure 3.5) and antihyperalgesic and antiallodynic effects in CFA-inflamed animals (Figure 3.6). Likewise, hPAP and bPAP had similar antinociceptive effects lasting three days and two days, respectively (Zylka et al., 2008). And, just like hPAP and bPAP

mediated antinociception, A_1R receptor activation was required for mPAP mediated antinociception. When combined with our biochemical results, this suggests that mPAP converts extracellular nucleotides to adenosine in vivo. Moreover, these data suggest a species-conserved function for human, bovine and mouse PAP as an ectonucleotidase.

Adenosine and A_1R agonists have potent and, in some studies, long-lasting (>24 h) analgesic effects in rodents and humans when injected peripherally or centrally (Hayashida et al., 2005; Lavand'homme and Eisenach, 1999; Sawynok, 2007). However, adenosine and A_1R agonists are not used clinically to treat chronic pain because of side-effects, including transient lower back pain (Belfrage et al., 1999; Eisenach et al., 2003), and motor paralysis when administered at high doses (Sawynok, 2007). Motor side-effects could be due to widespread expression of A_1R throughout the spinal cord, including relatively high-level expression in motor neurons (Reppert et al., 1991).

We did not observe motor paralysis at the highest doses of mouse and human PAP tested, despite the fact that PAP also works via A_1R activation (Figures 3.5, 3.6; (Zylka et al., 2008)). This could be due to the fact that, as an enzyme, the amount of adenosine produced by PAP is limited by substrate concentration. Thus, through catalytic restriction, PAP may produce sufficient amounts of adenosine to mediate antinociception but not enough adenosine to cause overt motor side-effects.

The resting CSF concentration of AMP in humans is 1.8 μM (Rodriguez-Nunez et al., 2000), well below the Km of mPAP and hPAP for AMP. Since this AMP concentration is below Km, PAP could produce linear increases in adenosine as the extracellular AMP concentration increases. This would allow PAP to dynamically generate adenosine over a

wide range of nucleotide concentrations. This could be relevant in chronic pain states where extracellular nucleotides are likely to be elevated (Holton, 1959; Tsuda et al., 2005).

Recombinant proteins, such as human growth hormone and interferons, are routinely used to treat a variety of human diseases and disorders (Dorr, 1993; Zucchini, 2008). We found that recombinant mPAP protein functions as a pH-dependent ectonucleotidase and has antinociceptive effects in an animal model of inflammatory pain. Unlike direct injections of adenosine and A_1R agonists which produce antinociception and motor side effects, mPAP injections *indirectly* elevate adenosine levels and produce antinociception without side-effects. Interestingly, other methods that indirectly elevate adenosine, such as using adenosine kinase inhibitors, also produce antinociception without motor side effects (Jarvis et al., 2002b; Keil and DeLander, 1992; Poon and Sawynok, 1995; Poon and Sawynok, 1998). Considering how readily recombinant mPAP and hPAP can be purified (Ostanin et al., 1994), and the fact that recombinant hPAP (fused to GM-CSF; also known as PA2024, a component of the Provenge/Sipuleucel-T immunotherapy) is safe to use in humans (Burch et al., 2000; Burch et al., 2004), recombinant PAP could be developed as a protein-based therapeutic for chronic pain. Moreover, it might be possible to further optimize PAP stability and kinetic parameters for therapeutic purposes, using site-directed mutagenesis and the PAP three-dimensional structure as a guide (Jakob et al., 2000; Ostanin et al., 1994; Porvari et al., 1994; Schneider et al., 1993).

3.5) Figures and Tables

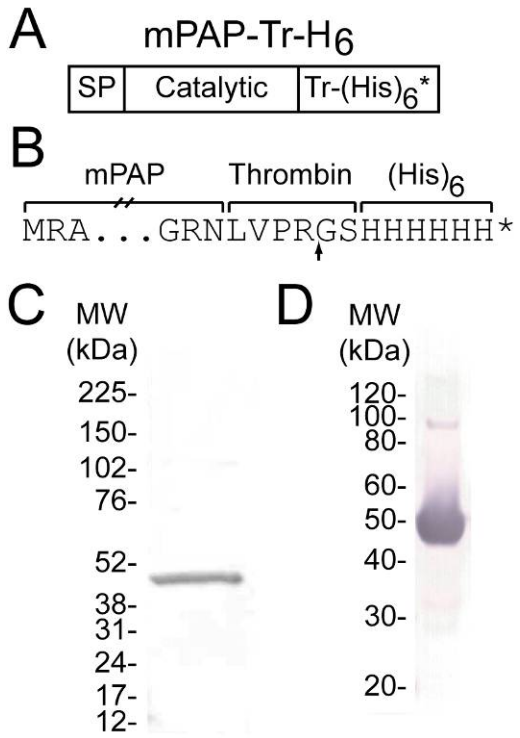

Figure 3.1. Purification of recombinant mPAP. (A) A thrombin cleavage site (Tr) followed by hexahistidine tag (H_6) and stop codon (*) were added to the C-terminus of the secretory isoform of mPAP. SP = signal peptide of mPAP. Map is not drawn to scale. (B) Amino acid sequence at the junction between the catalytic domain and Tr-H_6 tag. Arrow marks thrombin cleavage site. Asterisk marks stop codon. (C) GelCode blue-stained SDS-PAGE gel and (D) western blot of purified recombinant mPAP protein (1 μg and 5 μg, respectively). The western blot was probed with an anti-hexahistidine antibody. (Back to text)

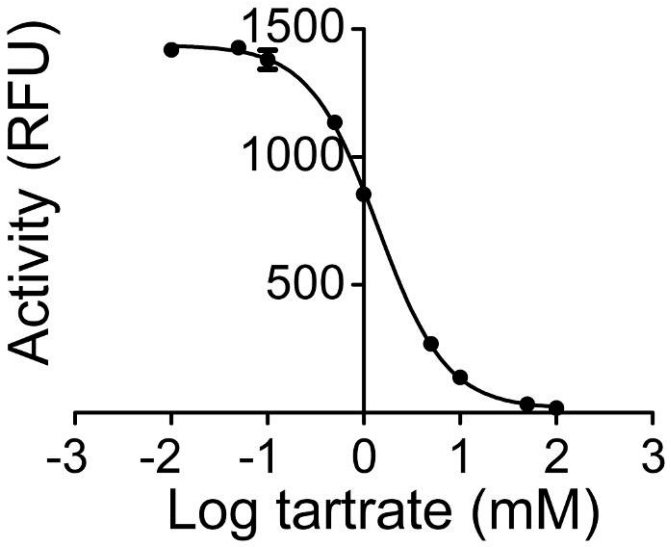

Figure 3.2. Inhibition of mPAP by L-(+)-tartrate. The indicated concentrations of L-(+)-tartrate were added to reactions (n=3 per concentration) containing mPAP (1 U/mL), 100 mM sodium acetate, pH 5.6 and the fluorescent acid phosphatase substrate DiFMUP. Relative fluorescence units (RFU). All data are presented as means ± s.e.m. Prism 5.0 (GraphPad Software, Inc) was used to generate curve. (Back to text)

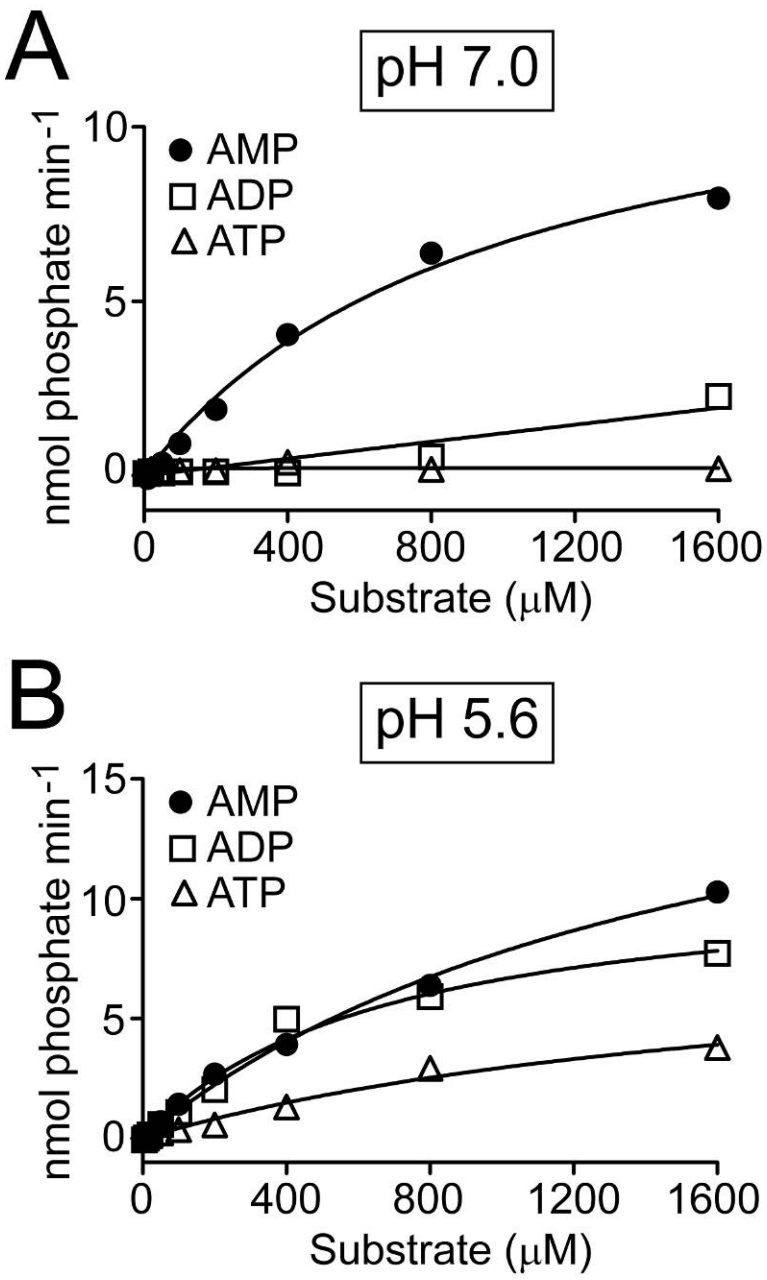

Figure 3.3. mPAP dephosphorylates purine nucleotides in a pH-dependent manner. Plot of initial velocity at the indicated concentrations of AMP, ADP and ATP at (A) pH 7.0 and (B) pH 5.6. Reactions (n=3 per point) were stopped after 3 min. Inorganic phosphate was measured using malachite green. All data are presented as means ± s.e.m. Error bars are obscured due to their small size. (Back to text)

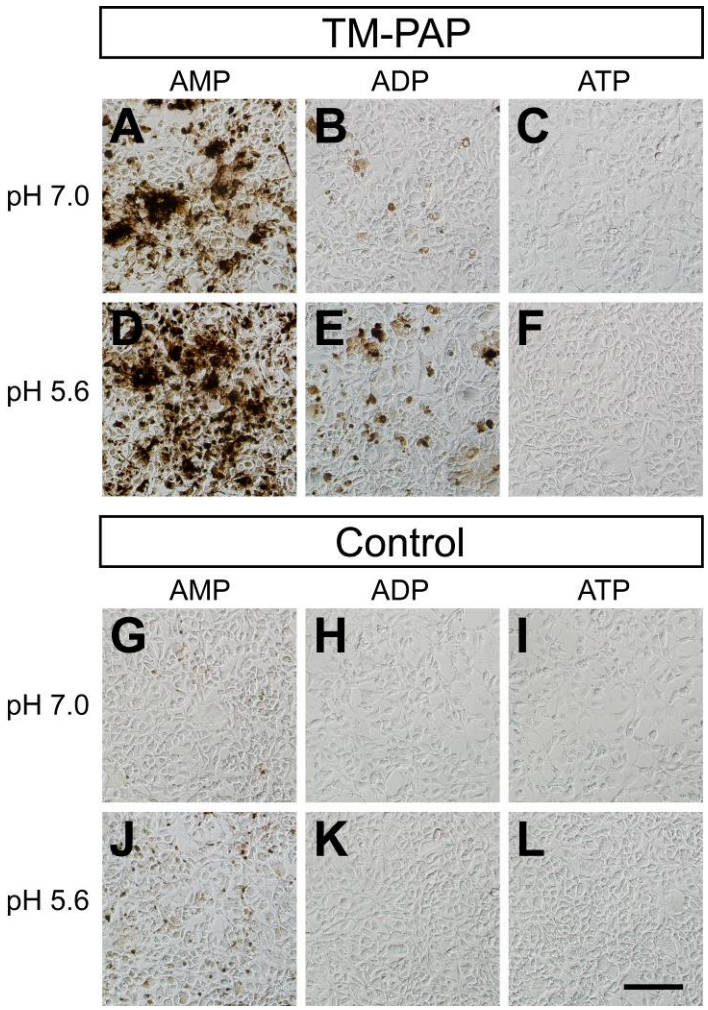

Figure 3.4. TM-PAP dephosphorylates extracellular purine nucleotides in a pH-dependent manner. HEK 293 cells were transfected with expression vectors containing (A-F) mouse TM-PAP or (G-L) the fluorescent protein Venus as a control. Cells were then histochemically stained using AMP, ADP or ATP (each 6 mM) as substrate at pH 7.0 or pH 5.6. Cells were not permeabilized with detergent. Scale bar (bottom right panel), 50 μm for all panels. (Back to text)

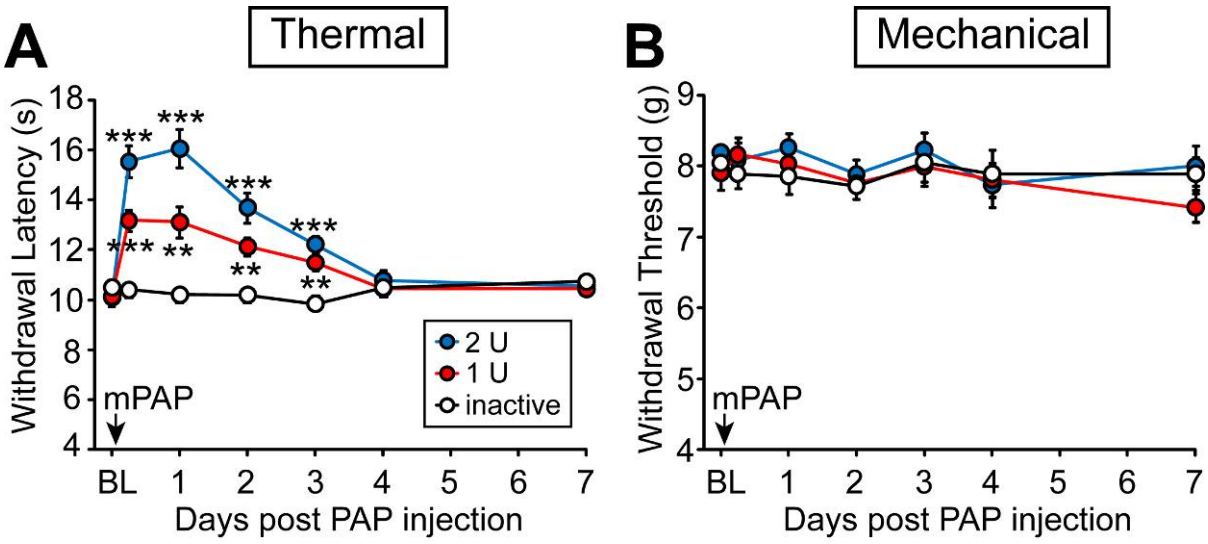

Figure 3.5. Dose-dependent antinociceptive effects of intrathecal mPAP. (A) Effects of increasing amounts of mPAP on paw withdrawal latency to a radiant heat source. (B) Paw withdrawal threshold to a semi-flexible tip mounted on an electronic von Frey apparatus. (A, B) BL=Baseline. Injection (i.t.) volume was 5 μL. n=8 wild-type mice were used per dose. There were significant differences over time between mice injected with heat-inactivated (0 U) mPAP and mice injected with active (1 U or 2 U) mPAP (Repeated measure two-way ANOVA; $P < 0.0001$ for each dose). Post-hoc paired t-tests were used to compare responses at each time point between mice injected with active mPAP to mice injected with heat-inactivated mPAP (** $P < 0.005$; *** $P < 0.0005$). For the heat-inactivated mPAP control, the protein concentration was equivalent to the maximum 2U dose of mPAP (1.1 mg/mL). All data are presented as means ± s.e.m. (Back to text)

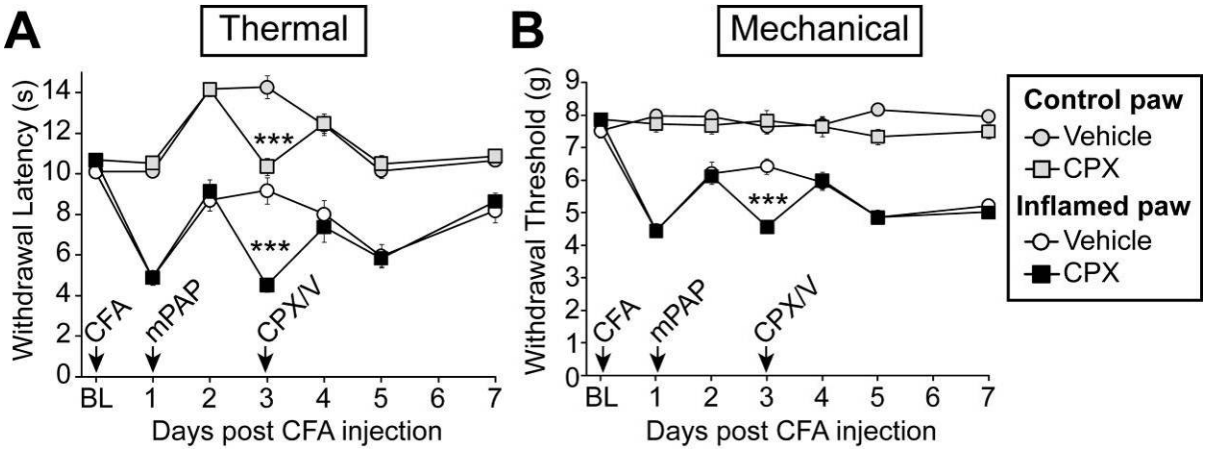

Figure 3.6. The antinociceptive effects of mPAP can be transiently inhibited with a selective A1R antagonist. Wild-type mice were tested for (A) noxious thermal and (B) mechanical sensitivity before (baseline, BL) and following injection of CFA (CFA-arrow) into one hindpaw. The non-inflamed hindpaw served as control. All mice were injected with active mPAP (mPAP-arrow; 2 U, i.t.). Two days later, half the mice were injected with vehicle (CPX/V-arrow, circles; intraperitoneal (i.p.); 1 hr before behavioral measurements) while the other half were injected with 8-cyclopentyl-1, 3-dipropylxanthine (CPX/V-arrow, squares; 1 mg/kg i.p.; 1 hr before behavioral measurements). There were significant differences over time between mice injected with vehicle and mice injected with CPX (Repeated measure two-way ANOVA; $P < 0.01$). Post-hoc paired t-tests were used to compare responses at each time point between vehicle (n=10) and CPX-injected mice (n=10); same paw comparisons. *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

CHAPTER 4

Prostatic Acid Phosphatase Reduces Pain Sensitization and TRPV1-dependent Thermal Sensitivity by Depleting PIP_2

ABSTRACT: Prostatic acid phosphatase (PAP) is an ectonucleotidase that inhibits noxious thermal sensitivity for days by generating adenosine and activating the A_1-adenosine receptor (A_1R). Currently, the mechanism through which PAP regulates sensitivity to noxious thermal stimuli is unknown. We found that sustained activation of A_1R by PAP inhibits signaling through the thermosensor TRPV1 by decreasing phosphatidylinositol 4,5-bisphosphate (PIP_2) levels in cells and dorsal root ganglia (DRG). In support of this, the thermal antinociceptive effects of PAP were blunted in *Trpv1*$^{-/-}$ mice and blocked when PIP_2 levels were pharmacologically restored. Additionally, PAP-mediated depletion of PIP_2 inhibits signaling through receptors that sensitize nociceptive neurons, including lysophosphatidic acid (LPA) and ATP receptors. Thus, PAP acts through A_1R to reduce TRPV1-dependent thermosensation and pro-nociceptive receptor sensitization by decreasing PIP_2. Moreover, our studies suggest selective depletion of PIP_2 in nociceptive circuits could be used to treat chronic pain before or after it is initiated.

This work has been submitted and is under review: Sowa, N.A., Vihko, P, and Zylka, M.J. *Manuscript submitted and in review*.

4.1) Introduction

Chronic pain affects more individuals than heart disease, diabetes and cancer combined (American Pain Foundation). Many of the analgesics that are used to treat pain act on subsets of nociceptive (pain-sensing) neurons located in the dorsal root ganglia or their postsynaptic targets in the dorsal spinal cord. Recently, we found that the transmembrane (TM) isoform of Prostatic Acid Phosphatase (PAP; also known as ACPP) is expressed in nociceptive neurons and functions as an ectonucleotidase (Zylka et al., 2008). Both the TM and secretory isoforms of PAP (S-PAP) dephosphorylate extracellular adenosine monophosphate (AMP) to adenosine (Sowa et al., 2009; Vihko, 1978; Zylka et al., 2008). Strikingly, we found that a single intrathecal (i.t.; intraspinal) injection of S-PAP had long-lasting antinociceptive effects that significantly outlasted the commonly used analgesic morphine (Sowa et al., 2009; Zylka et al., 2008). In addition, S-PAP had thermal antihyperalgesic and mechanical antiallodynic effects that lasted for three days in animal models of chronic inflammatory pain and nerve injury-induced neuropathic pain. All of these antinociceptive effects were completely eliminated in $A_1R^{-/-}$ mice indicating that S-PAP generates adenosine in vivo and inhibits nociception by activating A_1R over a sustained time period.

Although adenosine and A_1R agonists have well-studied antinociceptive effects when administered to rodents and humans (Eisenach et al., 2003; Sawynok, 2006), it is currently not known how acute or sustained A_1R activation regulates nociception at the molecular level. A_1R is a $G\alpha_{i/o}$-coupled receptor whose activation leads to inhibition of adenylate cyclase, and hence inhibition of protein kinase A (PKA). In addition, A_1R stimulation activates phospholipase C (PLC; including PLCβ3) via G$\beta\gamma$ proteins (Jacobson and Gao,

2006; Murthy and Makhlouf, 1995a). Acute A_1R activation can also inhibit neurotransmitter release from nociceptive neurons and inhibit postsynaptic spinal cord neurons (Lao et al., 2001; Li and Perl, 1994). While such a mechanism could account for some aspects of A_1R-mediated antinociception, inhibition of neurotransmission does not readily explain why sustained A_1R activation by PAP *selectively* inhibits noxious thermal sensitivity without affecting mechanical sensitivity in naïve mice (Sowa et al., 2009; Zylka et al., 2008). In turn, this selectivity suggests PAP might regulate thermal nociception by acting through a specific thermosensory channel or mechanism.

The capsaicin and noxious heat receptor TRPV1 is expressed in DRG neurons and functions as a thermosensor in vivo (Caterina et al., 2000b; Caterina et al., 1997; Davis et al., 2000b). These findings, combined with our observation that PAP and TRPV1 are co-expressed in DRG neurons (Zylka et al., 2008), suggested PAP might regulate thermal sensitivity through TRPV1. Using cell-based and behavioral assays, we found that sustained activation of A_1R by PAP led to PLC-mediated depletion of the phosphoinositide PIP_2. This reduction in PIP_2 inhibited TRPV1 activity and reduced thermosensation in vivo (Figure 4.1). Our in vivo findings are consistent with several in vitro studies showing that TRPV1 requires PIP_2 to function (reviewed in (Rohacs et al., 2008)). Additionally, we found that PAP inhibited signaling through diverse pro-nociceptive G protein-coupled receptors (GPCRs) by activating A_1R and depleting PIP_2 (Figure 4.1). Altogether, our studies suggest that many of the potent antinociceptive effects of PAP are mediated by sustained A_1R activation followed by depletion of PIP_2. Moreover, our studies are the first to demonstrate a role for PIP_2 in the modulation of nociception in vivo.

4.2) Materials and Methods

All procedures and behavioral experiments involving vertebrate animals were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

4.2.1) Histology

Enzyme histochemistry and immunofluorescence staining was performed as described (Zylka et al., 2005; Zylka et al., 2008).

4.2.2) Molecular Biology

Full-length expression constructs for mouse TM-PAP (nt 64- 1314 from GenBank accession # NM_207668) and human TM-PAP (nt 51-1304 from GenBank accession # BC007460) were generated by RT-PCR amplification using C57BL/6 mouse trigeminal cDNA or human placental cDNA (Clontech) as template and Phusion polymerase. The red fluorescent protein mCherry was then fused in-frame to the C-terminus of all TM-PAP constructs. Mouse TM-PAP(H12A) was generated by PCR-based mutagenesis using mouse TM-PAP as template (His12 corresponds to His43 of the mPAP preprotein). This active site mutant was previously described and lacks catalytic activity (Figures 4.5, 4.6) (Ostanin et al., 1994; Schneider et al., 1993). All constructs have a Kosak consensus sequence, were cloned into pcDNA3.1 and were sequence verified. We obtained additional constructs from others (see Acknowledgments). We confirmed that adenosine receptors were expressed in Rat1 fibroblasts by RT-PCR (A_1R primers: 5' CATTGGGCCACAGACCTACT and 5' GGCAGAAGAGGGTGATACA).

4.2.3) Calcium Imaging

Rat1 fibroblasts were grown on glass bottom culture dishes (MatTek Corp, P35G-0-10-C) in DMEM containing 10% Fetal Bovine Serum, 100 U/mL penicillin and 100 μg/mL streptomycin and transfected with Lipofectamine Plus (Invitrogen) according to manufacturer's protocol. The total amount of DNA per well was adjusted to 1 μg by adding pcDNA 3.1 as carrier. Following transfection (18-24 hours), cells were loaded for one hour at room temperature with 2 μM Fura-2 AM (Invitrogen, F-14185) in Hank's Buffered Salt Solution (HBSS + Calcium and Magnesium) assay buffer (HBSS + 9 mM HEPES + 11 mM D-Glucose + 0.1% fatty-acid free BSA, pH 7.4). Cells were then washed 3 times with HBSS assay buffer and sat for at least 30 min prior to imaging. A Nikon TE2000U microscope and Sutter DG4 light source were used to image calcium responses (excitation 340 nm / 380 nm; emission 510 nm). Cells were stimulated with 1 μM capsaicin, 100 nM LPA, 1 U/mL thrombin, 10 μM ATP or 1 μM bradykinin for 1-5 min, washed in HBSS assay buffer for 1 min, then stimulated with 0.006% SDS to evoke maximal calcium responses for normalization. We did not use ionomycin to normalize responses because this calcium ionophore activates Ca^{2+}-dependent PLC enzymes. As a result, the magnitude of the ionomycin-induced Ca^{2+} influx is also proportional to PIP_2 levels in cells.

Calcium responses were normalized by calculating the area under the curve (AUC) during ligand stimulation for each cell, and then dividing by the maximum SDS-evoked calcium response in each cell (Figure 4.3). These values were averaged over all cells for a given condition and then normalized relative to untransfected cells in the same field of view or relative to control cells (with the untransfected or control cell response set to 100%).

For thapsigargin experiments, HBSS assay buffer lacking Ca^{2+} and containing 1 mM EGTA was used to eliminate extracellular calcium. 10 μM thapsigargin was added for 5 min, the cells were then washed for 2 min with HBSS assay buffer, and stimulated with 0.006% SDS. For PTX experiments, Rat1 fibroblasts were incubated for 18 hours with 500 ng/mL PTX prior to loading with Fura-2 AM and stimulation with 100 nM LPA. For experiments with adenosine and cannabinoid receptor antagonists, PLC inhibitor (U73122), PKC inhibitor (staurosporine), or PKA inhibitor (KT5720), cells were incubated with antagonists for 3-4 hours, loaded in the presence of antagonists/inhibitors with Fura-2 AM for one hour, and then stimulated with 100 nM LPA or 10 μM ATP.

For experiments with capsaicin, Rat1 fibroblasts were transfected with TRPV1-GFP alone or various constructs and then were stimulated with 1 μM capsaicin (from 100 mM stock in 100% DMSO, dissolved to final concentration in HBSS assay buffer) for 1 min, followed by a 5 min wash in HBSS assay buffer, then stimulation with 0.006% SDS. In some cases, cells were incubated with CPX (5 μM), KT5720 (1 μM), or staurosporine (100 nM) for 3 hours prior to stimulation with capsaicin.

4.2.4) PIP_2 Quantification

For quantification of PIP_2 in vitro, HEK293 cells or Rat1 fibroblasts were plated onto glass coverslips and transfected with the construct PLCδ-PH-GFP along with indicated constructs using Lipofectamine Plus (Invitrogen), according to manufacturer's protocol. 18-24 hours later, the cells were fixed with 4% paraformaldehyde (PFA)-PBS. Cells were imaged on a Leica TCS-NT confocal microscope. GFP fluorescence on the plasma membrane of cells compared to the cytoplasm was quantified using ImageJ (National

Institutes of Health) by taking cross-sectional averages of pixel intensity at the plasma membranes and dividing by the average of pixel intensity in the cytoplasm. Cells (n=30 to 70) were analyzed per condition.

For quantification of PIP_2 in DRG, age-matched, adult male C57BL/6 or *Pap*$^{-/-}$ mice were injected i.t. with 5 μL of 15% lidocaine + 50 U/mL hPAP (250 mU total) or 15% lidocaine alone. Lidocaine causes transient (5-20 min.) paralysis of both hindlimbs, permitting us to visually determine if each mouse received a successful i.t. injection (we only quantified PIP_2 levels in mice that showed transient bilateral paralysis). One day later, mice were sacrificed and L3-L6 DRGs were dissected bilaterally (n=8 ganglia / sample) and placed in PBS on ice. For each sample, DRG wet weight was determined then lipids were extracted and quantified using the $PI(4,5)P_2$ Mass ELISA Kit from Echelon (K-4500) following the manufacturer's protocol. PIP_2 levels were normalized by dividing by the wet weight of DRG tissue.

4.2.5) Injections and Drugs

For intrathecal drug delivery, 5 μL was injected into unanesthetized mice using the direct lumbar puncture method (Fairbanks, 2003). Human S-PAP (Sigma, P1774) and heat-inactivated S-hPAP were prepared as described previously (Zylka et al., 2008). 18:1 Lysophosphatidic acid (Avanti Polar Lipids, 857130) was dissolved in 0.9% ethanol and then diluted to final concentrations in either HBSS assay buffer (calcium imaging) or 0.9% saline (injections). Adenosine 5'-triphosphate (ATP, Sigma, A26209) was dissolved in either HBSS assay buffer (calcium imaging) or 0.9% saline (injections). Capsaicin (Sigma, 2028 – 1 mg) was dissolved in 0.9% saline/10% ethanol/0.5% Tween 80 and 5 μl was injected for

intrathecal delivery, while 20 μL was injected for intraplantar delivery. U73122 (Tocris, 1268) was first dissolved into DMSO, then further diluted in 0.9% saline for i.t. injection. The PI(4,5)P_2 Shuttle PIP Kit (Echelon, P-9045) was used to increase PIP_2 levels in vivo. PtdIns(4,5)P_2 di-C_{16} was first dissolved into 10% DMSO in 0.9% saline. Carrier 2 (Histone H1) was dissolved into 0.9% saline. Prior to injection, PIP_2 and Carrier 2 were mixed in a 1:1 molar ratio and incubated at room temperature for 15 min. Thrombin (Sigma, T4648) was first dissolved to 100 Units/mL in 0.1% BSA and further diluted in HBSS assay buffer to final concentrations. BK was dissolved to 1 mM in DMSO and further diluted in HBSS assay buffer to final concentrations. PTX (Sigma, P7208) and caffeine (Sigma, C0750) were dissolved in water. 8-Cyclopentyl-1,3-dimethylxanthine (CPT) (Sigma, C102), 8-Cyclopentyl-1,3-dipropylxanthine (CPX) (Sigma, C101), SCH58261 (Sigma, S4568), MRS1754 (Sigma, M6316), MRS 1523 (Sigma, M1809), staurosporine (Sigma, S4400), KT5720 (Tocris, 1288), and U73122 (Tocris, 1268) were dissolved in DMSO and further diluted in HBSS assay buffer to final concentrations.

4.2.6) Behavior

Pap$^{-/-}$ and *Trpv1*$^{-/-}$ (B6.129X1-*Trpv1*^{tm1Jul}/J) mice were backcrossed to C57BL/6 mice for at least 10 generations. Knockout mice were matched to C57BL/6 control animals for age and weight. For all other experiments male, C57BL/6 mice were purchased from Jackson Laboratories. Male, 2-4 month-old mice were used for all behavioral studies. All mice were acclimated to testing room, equipment, and experimenter for 1-3 days before behavioral testing. The experimenter was blind to genotype and drug treatment during

behavioral testing. Thermal and mechanical sensitivity were measured as described previously (Zylka et al., 2008).

For the LPA and ATP injection experiments in WT vs. *Pap*$^{-/-}$ mice, after taking baseline measurements, 5 μl of LPA (5 nmol) or 5 μl of ATP (100 nmol) was injected into all mice. Thermal and mechanical sensitivity were then measured 1, 2, 3, 4, 5, and 12 days following LPA or ATP injection.

For the S-hPAP injection followed by LPA and ATP injection experiments, after taking baseline measurements, 5 μl of heat-inactivated S-hPAP or 250 mU of S-hPAP was injected i.t. One day later, thermal and mechanical sensitivity were measured in all mice, and then 5 μl of LPA (5 nmol) or 5 μl ATP (100 nmol) was injected i.t. into all mice. Thermal and mechanical sensitivity were then measured 1, 2, 3, and 7 days following LPA or ATP injection.

For the nerve injury experiment, heat-inactivated S-hPAP or 250 mU of S-hPAP was injected i.t. and thermal and mechanical sensitivity were measured 6 hours later. The next day the spared nerve injury (SNI) model of neuropathic pain was performed as described (Shields et al., 2003), and thermal and mechanical sensitivity was measured 1, 2, and 7 days post injury.

For the inflammatory pain study in WT and *Trpv1*$^{-/-}$ mice, after taking baseline measurements, 20 μL of complete Freund's adjuvant (CFA, MP Biochemicals, 642851) was injected into one hindpaw, centrally beneath glabrous skin using a 30G needle. One day later, heat-inactivated S-hPAP or 250 mU S-hPAP was injected i.t. Thermal and mechanical sensitivity were measured 1, 2, 3, and 4 days following S-hPAP injection.

For the capsaicin experiments, mice were acclimated to a plexiglass chamber for at least 30 minutes prior to capsaicin injection. For intraplantar experiments, 20 μl of 0.1 mg/ml capsaicin in 0.9% saline, 10% ethanol, 0.5% Tween-80 was injected (2 μg total) into the hindpaw, and the amount of time spent licking or biting the paw was measured for the first 2 min following injection. For intrathecal experiments, 5 μl of 0.1 mg/ml capsaicin (0.5 μg total) was injected by acute lumbar puncture and the amount of time spent licking or biting the caudal half of the body was measured for the first 5 min following injection.

For the PLC inhibitor (U73122) experiment, after taking baseline measurements 250 mU of S-hPAP was injected i.t. into two groups of WT C57BL/6 mice, while a third group of mice was not injected. Thermal sensitivity was measured 1 day later. Two days after S-hPAP injection, U73122 (5.4 nmol) or vehicle (20% DMSO in 0.9% saline) was injected i.t. and thermal sensitivity was measured 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 7 hr, 24 hr, and 48 hr after injection.

For the PIP_2 injection experiment, after taking baseline measurements, 250 mU of S-hPAP was injected i.t. into two groups of WT C57BL/6 mice, while a third group of mice was not injected. Thermal sensitivity was measured 1 day later. Two days after S-hPAP injection, PIP_2 + Carrier 2 or Carrier 2 alone was injected i.t., and thermal sensitivity was measured 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 24 hr, and 48 hr after injection.

For PIP2 co-injection with LPA, after taking baseline measurements, mice were injected with either PIP2 + Carrier 2 or Carrier 2 alone and thermal and mechanical sensitivity were measured 1d, 2d, 3d, 4d, 5d, and 12d after injection.

4.3) Results

4.3.1) PAP acts through A_1R to Inhibit TRPV1 Receptor Activation

We previously found that secretory human PAP (S-hPAP) reduced noxious thermal sensitivity for three days in naïve mice by generating adenosine and activating A_1R (Zylka et al., 2008). Precisely how such sustained A_1R activation regulates thermal sensitivity is unknown. TRPV1 is a nonselective cation channel that can be activated by noxious thermal stimuli or capsaicin (Caterina et al., 1997), suggesting that PAP might reduce thermal sensitivity by inhibiting TRPV1. To test this hypothesis in a cell-based context, we transiently transfected Rat1 fibroblasts with TRPV1 or with TRPV1 and mouse transmembrane PAP (TM-PAP) then measured capsaicin-evoked Ca^{2+} influx using the Ca^{2+} indicator Fura-2 AM. We found that both the amplitude and duration of capsaicin-evoked Ca^{2+} influx was significantly reduced in cells transfected with TM-PAP relative to cells expressing TRPV1 alone (Figure 4.2A, 4.2B, 4.3). In contrast, capsaicin-evoked Ca^{2+} influx was not reduced in cells transfected with mouse TM-PAP(H12A), a phosphatase-dead mutant of TM-PAP (Figure 4.2B) (Ostanin et al., 1994; Schneider et al., 1993). Additionally, the A_1R-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX) blocked the effect of TM-PAP on capsaicin-evoked signaling (Figure 4.2B; Rat1 cells express A_1R, Figure 4.4). Collectively, these data suggest that TM-PAP reduces TRPV1 signaling through activation of A_1R. Note that all TM-PAP constructs were fused to the red fluorescent protein mCherry and were expressed at similar levels in Rat1 cells and HEK293 cells (used below), but only the H12A mutant lacked catalytic activity (Figures 4.5, 4.6).

Next, to test whether the thermal antinociceptive effect of PAP was dependent on TRPV1 activity in vivo, we injected S-hPAP (i.t.) into WT and *Trpv1*$^{-/-}$ mice then measured paw withdrawal latencies to a noxious thermal stimulus. There were no significant

differences at baseline between WT and *Trpv1*$^{-/-}$ mice when stimulating the hindpaw with radiant heat (Figure 4.2C), consistent with previous studies (Caterina et al., 2000b). Paw withdrawal latency significantly increased (relative to baseline) 30 min after injecting (i.t.) S-hPAP and remained elevated for three days in WT mice, as expected (Zylka et al., 2008). However, the effects of S-hPAP on thermal sensitivity were significantly blunted in duration (2 days; relative to baseline) and in magnitude ($p < 0.001$ by two-way ANOVA; relative to WT) in *Trpv1*$^{-/-}$ mice (Figure 4.2C). In contrast, S-hPAP was equally effective at reducing mechanical allodynia in WT and *Trpv1*$^{-/-}$ mice following CFA-induced inflammation (Figure 4.2D; black dashed line verses red dashed line), ruling out the trivial possibility that *Trpv1*$^{-/-}$ mice were less sensitive to all antinociceptive effects of S-hPAP. We were unable to compare the effects of S-hPAP on CFA-induced thermal hyperalgesia in WT and *Trpv1*$^{-/-}$ mice because *Trpv1*$^{-/-}$ mice do not develop thermal hyperalgesia following inflammation (Figure 4.2E), as previously found by others (Caterina et al., 2000b; Davis et al., 2000b). Collectively, these data suggest S-hPAP decreased thermal sensitivity, in part, through inhibition of TRPV1.

Considering that PAP is expressed in 19% of all TRPV1^{+} neurons (Zylka et al., 2008) and reduced TRPV1 activity, we hypothesized that deletion of PAP might enhance TRPV1 activity. To test this hypothesis, we injected the selective TRPV1 agonist capsaicin into the hindpaw of WT and *Pap*$^{-/-}$ mice then measured the amount of time spent licking the injected hindpaw (Caterina et al., 2000b). We also injected capsaicin i.t. then measured the amount of time spent licking the caudal half of the body (Mousseau et al., 1994). Following both intraplantar and i.t. injections, capsaicin-induced licking behavior was significantly greater in *Pap*$^{-/-}$ mice compared to WT controls (Figure 4.2F). These enhanced responses were not due

to a difference in the percentage of TRPV1^{+} DRG neurons between WT and *Pap*$^{-/-}$ mice (Table 4.1). Nor were these enhanced responses due to neuroanatomical differences between genotypes as nociceptive circuit anatomy was normal in *Pap*$^{-/-}$ mice (Zylka et al., 2008). Taken together, our cell-based and in vivo data suggest injected (S-) and endogenous (TM-) PAP reduce thermal and capsaicin sensitivity by inhibiting TRPV1.

4.3.2) Activation of A_1R by TM-PAP Depletes PIP_2

Our findings raised the question of how A_1R activation by PAP inhibits TRPV1 at a mechanistic level. A_1R stimulation inhibits PKA via pertussis toxin (PTX)-sensitive $G\alpha_{i/o}$-proteins. In addition, A_1R stimulation activates PLCβ3 via PTX-sensitive G$\beta\gamma$ subunits (Dickenson and Hill, 1998; Murthy and Makhlouf, 1995a). PLC enzymes then hydrolyze PIP_2 in the membrane to diacylglycerol (DAG) and inositol triphosphate (IP_3). These facts suggested sustained activation of A_1R by PAP might inhibit TRPV1 activity by inhibiting PKA, activating protein kinase C (PKC; via DAG), depleting intracellular calcium stores (via IP_3) or depleting PIP_2 (via PLC activation). Although TRPV1 can be modulated by PKA and PKC (Bhave et al., 2003; Bhave et al., 2002; Huang et al., 2006b), TM-PAP did not inhibit TRPV1 through PKA or PKC pathways (Figure 4.7A, 4.7B). Furthermore, TM-PAP did not deplete intracellular calcium stores (Figure 4.7C).

TRPV1 can also be directly modulated by PIP_2 and this modulation is affected by capsaicin concentration and extracellular Ca^{2+} (Klein et al., 2008; Lishko et al., 2007; Liu et al., 2005; Lukacs et al., 2007; Prescott and Julius, 2003; Rohacs et al., 2008; Stein et al., 2006; Yao and Qin, 2009). At high capsaicin concentrations (1-10 μM) and in the presence of extracellular Ca^{2+}, PIP_2 is required for TRPV1 channel activity while depletion of PIP_2

desensitizes the channel (our cell-based assays above were performed with 1 μM capsaicin in the presence of extracellular Ca^{2+}). This requirement for PIP_2 suggested TM-PAP might inhibit TRPV1 activity by activating A_1R in a sustained manner then deplete PIP_2.

To test this possibility, we quantified the levels of PIP_2 in cells using the PIP_2 biosensor PLCδ-PH-GFP (Varnai and Balla, 1998). When PIP_2 levels are high, PLCδ-PH-GFP is primarily localized to the plasma membrane (PM). When PIP_2 is depleted, PLCδ-PH-GFP translocates from the membrane to the cytosol. This translocation can be quantified by measuring the GFP signal intensity on the PM relative to the cytosol (expressed as a ratio PM/Cytosol). We used HEK293 cells for these experiments because this biosensor was difficult to visualize in Rat1 fibroblasts (although we reproduced our key finding in Rat1 cells; Figure 4.9). In HEK293 cells expressing only PLCδ-PH-GFP, the majority of the GFP signal was in the PM, giving a PM/cytosol ratio of 3.43 ± 0.35 (Figure 4.8A, 4.8E). In contrast, PLCδ-PH-GFP was redistributed to the cytosol in cells co-transfected with TM-PAP or PLCβ3 (PM/cytosol ratio of 1.60 ± 0.06 and 1.70 ± 0.9, respectively) (Figure 4.8B, 4.8E). This finding suggested TM-PAP and PLCβ3 deplete PIP_2 to a similar extent. Importantly, the A_1R antagonist CPX blocked the TM-PAP mediated redistribution of PLCδ-PH-GFP to the cytosol (Figure 4.8C, 4.8E). In addition, the PLC inhibitor U73122 blocked TM-PAP-mediated PLCδ-PH-GFP redistribution (Figure 4.8E). Further, the TM-PAP- and PLCβ3-mediated redistribution of PLCδ-PH-GFP was blocked by overexpressing phosphatidylinositol-4-phosphate-5-kinase (PIPK; Figure 4.8D, 4.8E). PIPK dramatically increases PIP_2 levels in transfected cells (Lin et al., 2005; Milosevic et al., 2005), suggesting TM-PAP- and PLCβ3 alter PLCδ-PH-GFP membrane localization by depleting PIP_2.

4.3.3) TM-PAP Reduces TRPV1 Receptor Signaling by Depleting PIP_2

Next, we genetically manipulated PIP_2 levels to determine if increasing or decreasing PIP_2 affected capsaicin-evoked Ca^{2+} responses. Both TM-PAP and PLCβ3 deplete PIP_2 to a similar extent (Figure 4.8E) but only PLCβ3 hydrolyzes PIP_2 directly. Likewise, we found that both TM-PAP and PLCβ3 reduced capsaicin-evoked Ca^{2+} responses to a similar extent (Figure 4.8F), suggesting indirect or direct depletion of PIP_2 was sufficient to reduce TRPV1 activity. Similarly, others found that capsaicin-evoked responses through TRPV1 could be inhibited by selectively depleting PIP_2 with a rapamycin-inducible PIP_2 phosphatase (Klein et al., 2008; Yao and Qin, 2009). Conversely, increasing PIP_2 levels by overexpressing PIPK (which regenerates PIP_2) blocked the TM-PAP- and PLCβ3-mediated reduction in capsaicin-evoked Ca^{2+} (Figure 4.8F). This finding suggested signaling through TRPV1 was reduced as a direct result of PIP_2 depletion, consistent with the findings of others using cultured cells (Klein et al., 2008; Lishko et al., 2007; Liu et al., 2005; Lukacs et al., 2007; Rohacs et al., 2008; Stein et al., 2006; Yao and Qin, 2009). In addition, TM-PAP did not affect capsaicin evoked Ca^{2+} influx in cells expressing TRPV1Δ42(777-820) (Figure 4.10), a TRPV1 mutant that is missing a putative PIP_2 binding domain (Kim et al., 2008a; Kwon et al., 2007; Prescott and Julius, 2003). Taken together, these data show that TM-PAP reduces TRPV1 activity in vitro through sustained activation of A_1R and subsequent depletion of PIP_2.

4.3.4) PAP Regulates PIP_2 Levels in vivo

Considering that PAP regulated PIP_2 levels in cultured cells, we hypothesized PAP might also regulate PIP_2 levels in vivo. To test this hypothesis, we measured PIP_2 levels in lumbar (L)3-L6 DRG from wild-type (WT) mice, S-hPAP injected WT mice, and *Pap*$^{-/-}$

mice. We found that PIP_2 levels were inversely related to the amount of PAP activity such that, relative to WT mice, PIP_2 levels were reduced following S-hPAP injection and increased when PAP was deleted (Figure 4.11A).

Since our data suggested that PAP depletes PIP_2 by activating PLC, we next evaluated whether the thermal antinociceptive effect of S-hPAP could be blocked using the PLC inhibitor U73122. This inhibitor was previously injected i.t. to block PLC activation by a delta opioid receptor ligand (Narita et al., 2000). Indeed, i.t. injection of U73122 transiently blocked the thermal antinociceptive effects of S-hPAP, providing evidence that S-hPAP acted through PLC to reduce thermal sensitivity in vivo (Figure 4.11B, 4.11C).

To directly determine if S-hPAP reduced thermal sensitivity by depleting PIP_2, we replenished PIP_2 in lumbar spinal cord and DRG through i.t. injection of PIP_2 complexed with a carrier molecule. This PIP_2 shuttle was previously used to increase PIP_2 levels in cultured cells (Ozaki et al., 2000), but to our knowledge has never been used in vivo. Strikingly, i.t. injection of PIP_2 (complexed with carrier) transiently reversed S-hPAP-mediated thermal antinociception whereas carrier alone had no effect (Figure 4.11D, 4.11E). In addition, PIP_2 caused modest thermal hyperalgesia in control animals injected with PIP_2, suggesting thermal sensitivity can be transiently enhanced when PIP_2 levels are elevated above normal levels. Importantly, the magnitude of this effect on thermal sensitivity in control animals was smaller than in animals that were injected with S-hPAP and PIP_2. This argues that PIP_2 replenishment was sufficient to block the thermal antinociceptive effect of S-hPAP independent of how PIP_2 affects thermal sensitivity in control animals.

We previously found that PAP activates A_1R over a sustained three-day time period in vivo (Sowa et al., 2009; Zylka et al., 2008). This finding combined with our present data,

suggests a mechanism (Figure 4.11F) where (1) TM- and S-hPAP function as ectonucleotidases to generate adenosine. Adenosine then stimulates (2) A_1R in a sustained fashion, followed by (3) PLC activation and (4) PIP_2 hydrolysis. As a result, the amount of PIP_2 available to regulate TRPV1 activity (5) is reduced, leading to decreased channel activation and decreased noxious thermal sensitivity. In addition, our data suggest a novel approach for reducing PIP_2 levels in vivo for days by pharmacologically or genetically controlling PAP activity.

4.3.5) PAP Inhibits Pro-nociceptive LPA Receptor Signaling by Acting through A_1R to Deplete PIP_2

Diverse chemicals are released upon injury and inflammation and sensitize nociceptive neurons, in many cases, by activating pro-nociceptive GPCRs (Hucho and Levine, 2007b; Julius and Basbaum, 2001). Activation of many of these receptors leads to downstream activation of PLC, which hydrolyzes PIP_2 into the second messengers DAG and IP_3. Considering that pro-nociceptive receptors require PIP_2 for effective signaling and PAP can deplete PIP_2 upon sustained A_1R activation, we hypothesized that PAP might also reduce signaling through pro-nociceptive receptors. In turn, reduced signaling might reduce sensitization following receptor activation. To evaluate this possibility, we first focused on LPA receptors because LPA sensitizes nociceptive neurons, has long-lasting pro-nociceptive effects when injected intrathecally (i.t.) and is implicated in neuropathic pain mechanisms (Elmes et al., 2004; Inoue et al., 2004; Park and Vasko, 2005). In addition, LPA receptors are coupled to $G\alpha_{q/11}$ proteins, signal through PLC activation and evoke calcium (Ca^{2+})

influx when stimulated in many cell-types, including Rat1 fibroblasts (which endogenously express LPA receptors) (Kelley et al., 2006; Mills and Moolenaar, 2003).

First, we transiently transfected Rat1 fibroblasts with mouse TM-PAP then measured LPA-evoked Ca^{2+} influx. We found that the amplitude and duration of LPA-evoked Ca^{2+} influx were significantly reduced in cells transfected with TM-PAP relative to untransfected cells in the same field of view (Figures 4.12A, 4.12D). This "PAP effect" was species-conserved as cells transfected with human TM-PAP (TM-hPAP) were also less responsive to LPA stimulation (Figures 4.12B, 4.12D). In contrast, LPA-evoked Ca^{2+} influx was not reduced in cells transfected with catalytically inactive mouse TM-PAP(H12A) (Figures 4.12C, 4.12D).

Next, to determine if TM-PAP inhibited LPA-evoked signaling by generating adenosine and activating A_1R, we assessed whether PTX (an inhibitor of $G\alpha_{i/o}$-coupled receptors) or adenosine receptor antagonists could block the effect of TM-PAP on LPA-evoked signaling. We found that PTX completely blocked the PAP effect, as evidenced by no significant differences between untransfected cells and TM-PAP transfected cells that were treated with PTX (Figure 4.12E). Additionally, the PAP effect was blocked by the A_1/A_{2B} adenosine receptor antagonist caffeine (Caff) and by two different A_1R-selective antagonists: CPT (8-cyclopentyl-1,3-dimethylxanthine) and CPX (Figure 4.12E). In contrast, selective antagonists of all other adenosine receptors ($A_{2A}R$: SCH 58261; $A_{2B}R$: MRS 1754; A_3R: MRS 1523) did not block the PAP effect (Figure 4.13).

Next, we genetically manipulated PIP_2 levels to determine if increasing or decreasing PIP_2 affected LPA-evoked Ca^{2+} responses. As found above, both TM-PAP and PLCβ3 depleted PIP_2 to a similar extent (Figure 4.8E) but only PLCβ3 hydrolyzes PIP_2 directly.

Likewise, both TM-PAP and PLCβ3 reduced LPA-evoked Ca^{2+} responses to a similar extent (Figure 4.12E), suggesting indirect or direct depletion of PIP_2 was sufficient to reduce signaling. Conversely, increasing PIP_2 levels by overexpressing PIPK blocked the TM-PAP- and PLCβ3-mediated reduction in LPA-evoked Ca^{2+} responses (Figure 4.12E). This finding indicated that TM-PAP and PLCβ3 inhibit LPA receptor signaling as a direct result of PIP_2 depletion. Additionally, the inhibitory effect of TM-PAP on LPA-evoked signaling was blocked with the PLC inhibitor U73122 (Figure 4.12E), demonstrating that TM-PAP acts through PLC to deplete PIP_2.

As found above with TRPV1, TM-PAP did not reduce LPA signaling by acting through other pathways that are downstream of A_1R, including $G\alpha_{i/o}$-mediated inhibition of PKA, DAG-mediated PKC activation or IP_3-mediated depletion of intracellular calcium stores (Figures 4.7C, 4.14A, 4.14B).

Collectively these data support a mechanism (Figure 4.12F) where (1) TM- and S-hPAP function as ectonucleotidases to generate adenosine. Adenosine then stimulates (2) A_1R in a sustained fashion, followed by (3) PLC activation and (4) PIP_2 hydrolysis. This reduces the amount of PIP_2 available for (5) $G\alpha_{q/11}$/PLC-mediated LPA receptor signaling. With less PIP_2 available, there is less IP_3 (and DAG) generated following LPA receptor stimulation, resulting in smaller LPA-evoked Ca^{2+} responses.

4.3.6) TM-PAP Reduces Signaling through Several Pro-nociceptive GPCRs

To determine if this mechanism generalized to other classes of pro-nociceptive $G\alpha_{q/11}$-coupled receptors, we assessed whether TM-PAP could reduce Ca^{2+} influx from protease activated receptors (using the ligand Thrombin, Thr), purinergic receptors (using the

nonselective P2Y ligand ATP), and bradykinin (BK) receptors. Importantly, activation of these receptors evokes transient Ca^{2+} influx in Rat1 cells and has pro-nociceptive effects in vivo (Burnstock, 2007; Dale and Vergnolle, 2008; Kelley et al., 2006; Sawynok, 2006; Wang et al., 2006). Strikingly, Ca^{2+} responses induced by Thr, ATP and BK were reduced in TM-PAP transfected cells relative to untransfected cells, and these reductions were blocked by the A_1R antagonist CPX or by overexpression of PIPK (Figure 4.15). Collectively, these data suggest that TM-PAP interferes with signaling through multiple pro-nociceptive receptors by activating A_1R and depleting PIP_2.

4.3.7) PAP Blocks LPA-, ATP- and Nerve Injury-induced Hyperalgesia and Allodynia

Since PAP reduced signaling through pro-nociceptive receptors by depleting PIP_2 in Rat1 fibroblasts and reduced PIP_2 in DRG in vivo, this finding suggested PAP might also reduce signaling through pro-nociceptive receptors in vivo. To test this possibility, we took advantage of the fact that both LPA and ATP produce long lasting (>7 day) thermal hyperalgesia and mechanical allodynia when injected i.t. (Inoue et al., 2004; Nakagawa et al., 2007). In comparison, S-hPAP has antinociceptive, antihyperalgesic and antiallodynic effects that last for three days when injected i.t. (Zylka et al., 2008). We reasoned that if S-hPAP were injected one day before ATP or LPA, we could ascertain whether S-hPAP directly reduced LPA- and ATP-evoked signaling in vivo by measuring LPA- and ATP-evoked hyperalgesia and allodynia on days four and eight; corresponding to one and five days (respectively) after the three day antinociceptive effects of PAP wore off.

First, we measured baseline (BL) noxious thermal sensitivity and mechanical sensitivity in three groups of WT mice. One day later, we injected S-hPAP (i.t.) into two of

the groups and heat inactivated S-hPAP into the third (control; catalytically dead) group. As previously found (Zylka et al., 2008), S-hPAP increased paw withdrawal latency to the noxious thermal stimulus but had no effect on mechanical sensitivity, whereas inactive S-hPAP had no effects on thermal or mechanical sensitivity (Figure 4.16A-D). One day later, we injected (i.t.) either 5 nmol LPA (Figure 4.16A, 4.16B) or 100 nmol ATP (Figure 4.16C, 4.16D). Both pro-nociceptive compounds produced long-lasting (8 day) thermal hyperalgesia and mechanical allodynia in the control mice. In contrast, these pro-nociceptive compounds had no or minimal effects in mice injected with S-hPAP. Specifically, the S-hPAP + LPA and S-hPAP + ATP injected mice did not develop thermal hyperalgesia or mechanical allodynia. In fact, their thermal and mechanical sensitivities were at or near baseline levels on days four and eight (i.e., after the antinociceptive effects of S-hPAP wore off). These data provide compelling evidence that PAP, via sustained A_1R activation and PIP_2 depletion, can reduce signaling through two distinct pro-nociceptive receptors in vivo.

Conversely, since *Pap*$^{-/-}$ mice had elevated levels of PIP_2 in lumbar DRG, we hypothesized LPA and ATP receptors might signal more effectively and produce greater sensitization when activated in these animals. Indeed, we found that the thermal hyperalgesia and mechanical allodynia induced by LPA and ATP were enhanced in *Pap*$^{-/-}$ mice relative to WT mice (Figure 4.17), suggesting these receptors signal more effectively when PIP_2 levels are elevated in vivo.

Since higher PIP_2 levels in DRG of *Pap*$^{-/-}$ mice led to increased LPA-induced hyperalgesia, we next asked if we could also enhance LPA-hyperalgesia in wild-type mice by transiently increasing PIP_2 in the DRG of these animals. To do this, we directly injected PIP_2 using the shuttle system described above. Importantly, i.t. injection of PIP_2 alone using this

system only affects thermal sensitivity for a period of one hour after injection (Figure 4.11E) and has no direct long-term effects on either thermal or mechanical sensitivity (Figure 4.18C, 4.18D). Strikingly, co-injection of LPA + PIP_2 + Carrier led to significantly greater thermal hyperalgesia and mechanical allodynia than that seen in mice injected with LPA + Carrier alone (Figure 4.18A, 4.18B). Since PIP_2 itself is not having any effect over such a long period of time, this likely reflects increased LPA receptor signaling due to increased PIP_2 in the DRG at the time of receptor activation. This suggests that the activity of the nociceptors can be dynamically modulated by changes in cellular PIP_2 levels.

While LPA and ATP are pro-nociceptive when injected i.t., these chemicals may not fully model the complex pathologies that are associated with chronic pain conditions. To determine if reducing PIP_2 levels with S-hPAP had a more generalized effect on the signals that initiate chronic pain, we tested the effects of S-hPAP in the spared nerve injury (SNI) model of neuropathic pain. Strikingly, i.t. injection of S-hPAP prior to nerve injury eliminated thermal hyperalgesia and greatly reduced mechanical allodynia for eight days compared to mice injected with inactive S-hPAP (Figure 4.19A, 4.19B). This finding suggests that lowering PIP_2 levels with S-hPAP can interfere with the signals that initiate neuropathic pain.

4.4) Discussion

We previously found that PAP had potent antinociceptive effects that were entirely dependent on A_1R activation, including inhibitory effects on noxious thermal and mechanical sensitivity (Sowa et al., 2009; Zylka et al., 2008). How PAP and A_1R activation regulated sensitivity to such stimuli at the molecular level was unknown. By using in vitro and in vivo

assays to study two distinct classes of proteins that require PIP_2 for activity (TRPV1 and GPCRs), our study provides complementary support that several of the antinociceptive effects of PAP are due to sustained A_1R activation followed by PLC-mediated PIP_2 depletion. In particular, depletion of this important phosphoinositide has inhibitory effects on signaling through the thermosensor TRPV1 and blocks sensitization through several pro-nociceptive $G\alpha_{q/11}$-coupled GPCRs (Figures 4.1, 4.11F, and 4.12F). The effects of PIP_2 depletion have not been studied following sustained A_1R activation, presumably because the genetic tools to measure and manipulate PIP_2 only recently became available (Varnai et al., 2007). Lastly, our studies are the first to demonstrate a role for PIP_2 in the modulation of pain sensitivity in vivo.

Numerous studies found that TRPV1 can be modulated by PIP_2 in vitro, with effects that are dependent on capsaicin concentration and extracellular calcium (reviewed in (Rohacs et al., 2008)). At low capsaicin concentrations and in the absence of extracellular Ca^{2+}, PIP_2 partially inhibits TRPV1 (Prescott and Julius, 2003). In comparison, at high capsaicin concentrations and in the presence of extracellular Ca^{2+}, PIP_2 is required for TRPV1 activation and PIP_2 depletion inhibits TRPV1 through desensitization (Klein et al., 2008; Lishko et al., 2007; Liu et al., 2005; Lukacs et al., 2007; Stein et al., 2006; Yao and Qin, 2009). All these studies examined PIP_2 modulation of TRPV1 using excised patches or cultured cells. This seemingly complex relationship between PIP_2 levels and TRPV1 activity in cell-based (in vitro) assays makes it difficult to predict how PIP_2 levels might affect TRPV1 in vivo. In our present study, we increased or decreased PIP_2 levels for extended periods of time in vivo by manipulating PAP activity and then measured how these changes affected TRPV1-dependent behaviors. We found that elevated PIP_2 levels enhanced TRPV1

activity, as evidenced by increased capsaicin-evoked licking in *Pap*$^{-/-}$ animals. Conversely, decreasing PIP_2 levels reduced TRPV1 activity, as evidenced by reduced TRPV1-dependent thermal sensitivity following PAP injections. Furthermore, acutely restoring PIP_2 levels, by pharmacologically inhibiting PLC activation with U73122 (Figure 4.11B, 4.11C) or by injecting PIP_2, blocked the thermal antinociceptive effect of S-hPAP. Injection of PIP_2 in naïve mice without prior S-hPAP injection led to a small but significant decrease in thermal withdrawal latencies (Figure 4.11D, 4.11E), suggesting elevated levels of PIP_2 produce thermal hyperalgesia. Mechanistically, this could reflect enhanced activity of temperature sensitive ion channels like TRPV1. Taken together, our findings indicate that the net effect of PIP_2 is to enhance TRPV1-mediated thermal sensitivity in vivo. This is consistent with a recent study showing that TRPV1 activity was enhanced in vitro and in vivo through interactions with PIRT, a phosphoinositide-binding protein (Kim et al., 2008a). Our in vivo studies are more biologically-relevant than studies which exclusively use cultured cells. This includes DRG neurons, especially since the culturing process can change TRPV1 expression or activity (Shu and Mendell, 1999; Story et al., 2003b; Stucky et al., 2009).

Pro-nociceptive ligands sensitize TRPV1 by activating PKC, which is downstream of PLC activation (Bhave et al., 2003; Huang et al., 2006b). Considering that A_1R is also coupled to PLC enzymes (Jacobson and Gao, 2006; Murthy and Makhlouf, 1995a), why then does PAP inhibit TRPV1 activation upon A_1R activation, as we observed, instead of sensitizing TRPV1? This likely reflects differences in how pro-nociceptive GPCRs and A_1R couple to PLC enzymes and downstream signaling pathways. For example, pro-nociceptive receptors, like LPA receptors, are coupled to PLC isoforms via $G\alpha_{q/11}$ and $G\beta\gamma$ proteins and evoke a large but transient calcium influx and PKC activation upon sustained agonist

stimulation (Figure 4.12A, 4.12B) (Kelley et al., 2006; Mills and Moolenaar, 2003). In contrast, A_1R is coupled to PLC isoforms exclusively via Gβγ proteins and does not desensitize (as measured by CPX-sensitive antinociception) when activated for up to three days by PAP ectonucleotidase-generated adenosine (Sowa et al., 2009; Zylka et al., 2008). Moreover, long-term expression of TM-PAP or treatment of cells with S-hPAP did not alter baseline Ca^{2+} levels when compared to cells not exposed to PAP (Figure 4.3 and data not shown), suggesting sustained activation of A_1R by PAP does not detectably alter Ca^{2+} influx. Moreover, this suggests PAP activates PLC enzymes at a low level and for sustained periods of time. This low level activation is sufficient to deplete PIP_2 in an A_1R-dependent manner (Figure 4.8E) but is unlikely to produce sufficient amounts of DAG to activate PKC or to reduce intracellular calcium stores by producing IP_3. Indeed, we found that PAP did not inhibit TRPV1 activity by acting through PKC (Figure 4.7A) and PAP did not reduce intracellular (IP_3-sensitive) Ca^{2+} stores (Figure 4.7C). Thus, acute, high-level PLC activation, as occurs following pro-nociceptive ligand stimulation, could readily account for why PLC activation sensitizes TRPV1 through PKC. Conversely, sustained, low-level PLC activation, as occurs following activation of A_1R by PAP, could account for why PLC activation inhibits TRPV1 through PIP_2 depletion.

Puntambekar and colleagues recently found that adenosine could bind directly to TRPV1 and inhibit its activity (Puntambekar et al., 2004). However, adenosine made by PAP is unlikely to inhibit TRPV1 directly considering that: 1). PIPK, an enzyme that generates PIP_2 intracellularly, blocked PAP inhibition of TRPV1; 2). the A_1R antagonist CPX blocked PAP inhibition of TRPV1 and 3). PAP had no inhibitory effects on Ca^{2+} influx

through TRPV1Δ42, a functional version of TRPV1 that lacks a putative PIP_2 binding domain (Kim et al., 2008a; Kwon et al., 2007; Prescott and Julius, 2003).

We found that most of the thermal antinociceptive effects of PAP were lost in *Trpv1*$^{-/-}$ mice while the mechanical antinociceptive effects of PAP were preserved (Figure 4.2C, 4.2D). This dissociation suggests most of the thermal antinociceptive effects of PAP are mediated through TRPV1, while the remaining thermal and mechanical antinociceptive effects of PAP are mediated by other channels or proteins. PIP_2 generally increases the activity of many ion channels while depletion of PIP_2 reduces channel activity (Suh and Hille, 2005). PIP_2 depletion also inhibits synaptic vesicle exocytosis (Di Paolo and De Camilli, 2006). Further studies will be needed to determine if additional antinociceptive effects of PAP are due to inhibition or modulation of other PIP_2 sensitive channels, proteins or mechanisms.

4.4.1) PAP Reduces Pro-nociceptive Receptor Signaling and Sensitization

Our findings with PAP and TRPV1 led us to test whether PAP could regulate other aspects of nociception, particularly sensitization, by depleting PIP_2. Diverse pro-nociceptive compounds sensitize nociceptors centrally and peripherally by activating $G\alpha_{q/11}$-coupled GPCRs (Hucho and Levine, 2007b; Julius and Basbaum, 2001; Woolf and Ma, 2007). This sensitization contributes to allodynia and hyperalgesia in chronic pain conditions. Our studies reveal that PAP inhibits signaling through multiple pro-nociceptive receptors in vitro by depleting PIP_2. With less PIP_2 available, there should be less DAG and IP_3 produced upon pro-nociceptive receptor stimulation to sensitize neurons via PKC/DAG-dependent and Ca^{2+}-dependent pathways. Indeed, we found that PAP completely blocked sensitization

caused by two long-lasting pro-nociceptive compounds (LPA and ATP) in vivo. The antinociceptive effects of PAP in sensitized states, including inflammatory and neuropathic pain (Sowa et al., 2009; Zylka et al., 2008), might similarly be due inhibition of pro-nociceptive GPCR signaling. Lastly, we found that PAP preemptively blocked thermal hyperalgesia and blunted mechanical allodynia following nerve injury. Thus, by inhibiting signaling through multiple pro-nociceptive receptors, PAP might more effectively be used to treat chronic pain, before or after it is initiated, when compared to antagonists that selectively target individual pro-nociceptive receptors.

4.4.2) PIP_2 Levels Affect Responses to Nociceptive Stimuli

All cells constitutively release low nanomolar concentrations of ATP that are then converted to ADP, AMP and adenosine by ectonucleotidases (Yegutkin, 2008; Zimmermann, 2000). Similarly, basal ATP release increases in DRG after nerve injury and following stimulation (Holton, 1959; Matsuka et al., 2008). PAP generates adenosine from extracellular nucleotides and is found on nociceptive neurons along with A_1R (Schulte et al., 2003; Zylka et al., 2008). This makes PAP well-localized to dynamically regulate PIP_2 levels as a function of the concentration of extracellular nucleotides, the activity levels of PAP and other ectonucleotidases, and ultimately the level of basal A_1R activation. As has been hypothesized for other systems (Boison, 2008), this “adenosine tone”, and as our studies suggest “phosphoinositide tone”, allows neural circuit activity to dynamically adjust as inputs change.

In addition, our studies suggest PIP_2 levels affect the extent to which pro-nociceptive signals—be they thermal, chemical (LPA, ATP, capsaicin) or pathological (inflammation,

nerve injury)—produce hyperalgesia, allodynia and nociceptive behaviors. We observed a direct correlation between PIP_2 levels and induced pain sensitivity—PAP knockout mice have elevated levels of PIP_2 in DRG and show enhanced sensitivity following LPA injection (Figure 4.17A, 4.17B), ATP injection (Figure 4.17C, 4.17D), capsaicin injection (Figure 4.2F), inflammation, and nerve injury (Zylka et al., 2008). Likewise, thermal sensitivity was transiently enhanced following direct intraspinal injection of PIP_2, and co-injection with PIP2 increased LPA-induced hyperalgesia (Figure 4.18A, 4.18B). Conversely, injection of PAP lowers PIP_2 levels in DRG and reduces sensitivity following LPA injection (Figure 4.16A, 4.16B), ATP injection (Figure 4.16C, 4.16D), inflammation, and nerve injury (Zylka et al., 2008). Taken together, our studies suggest selective depletion of PIP_2 in nociceptive circuits could provide a novel approach to preemptively block chronic pain before it is initiated as well as to treat chronic pain once it is established.

4.5) Figures and Tables

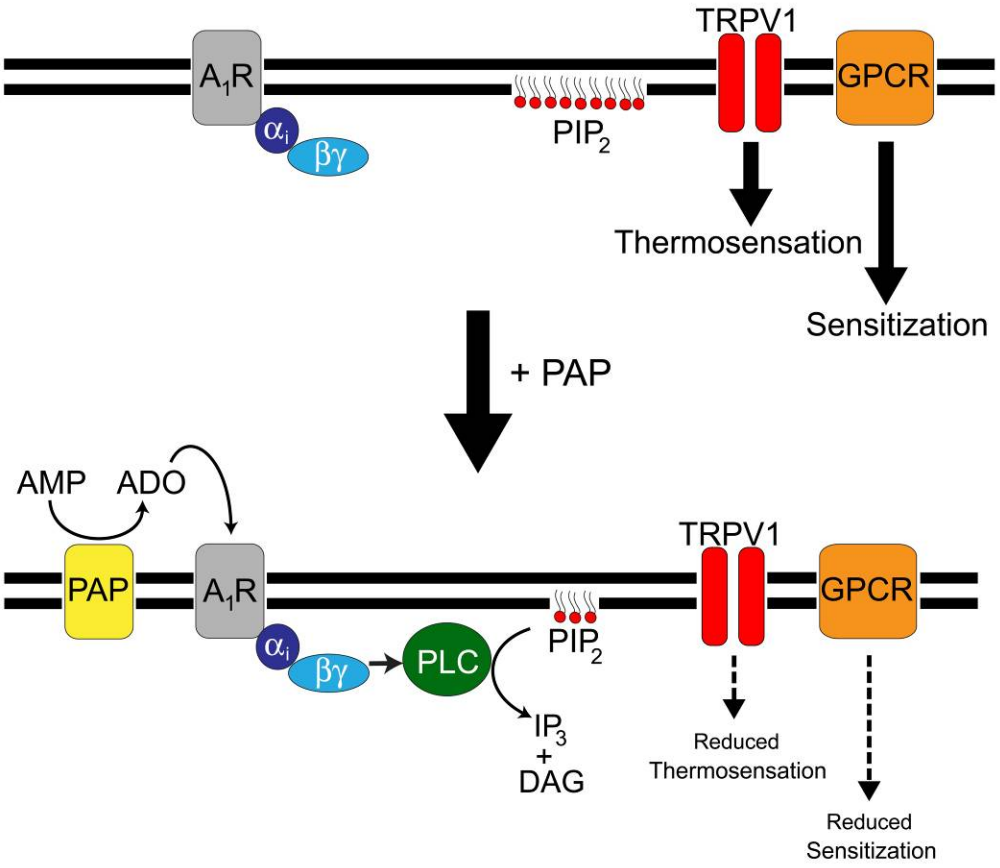

Figure 4.1. Proposed model—PAP reduces TRPV1-dependent noxious thermal sensitivity and pain sensitization by depleting PIP_2. PAP is an ectonucleotidase that generates adenosine (ADO) and activates A_1R for days without desensitizing. Sustained A_1R activation leads to phospholipase C (PLC) activation and PIP_2 depletion. Depletion of PIP_2 reduces noxious thermal sensitivity through TRPV1. Pro-nociceptive GPCRs sensitize nociceptive neurons when activated. Depletion of PIP_2 reduces signaling through pro-nociceptive GPCRs, thus reducing nociceptor sensitization. (Back to text)

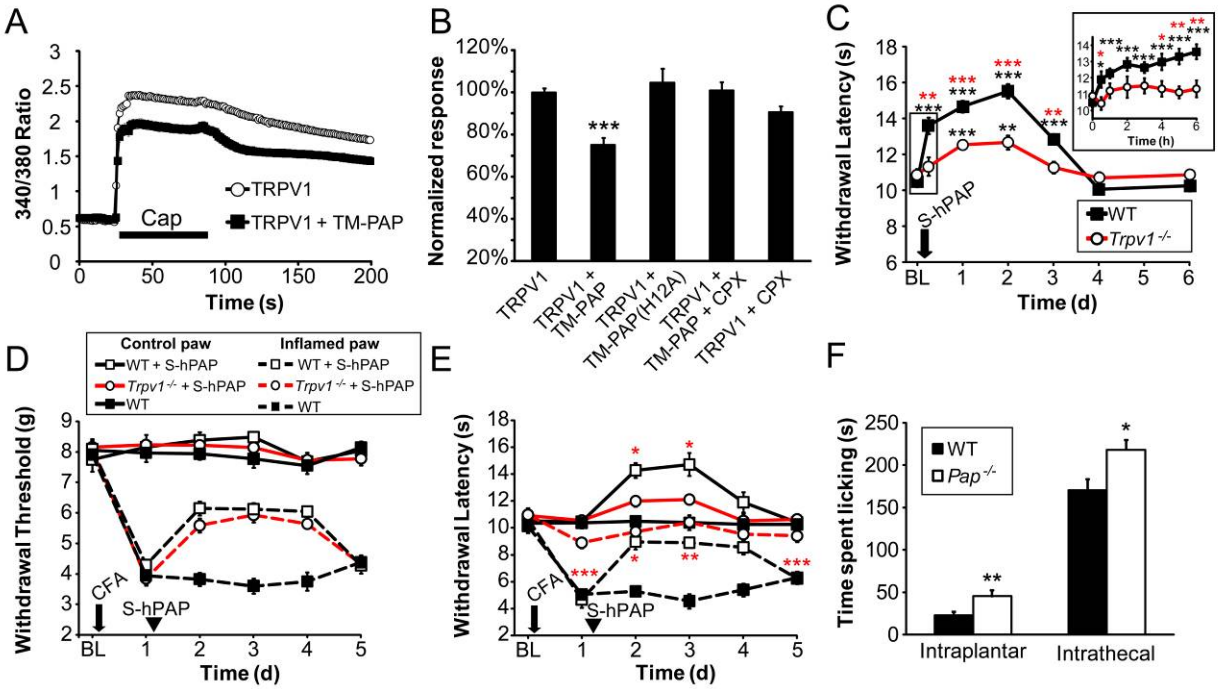

Figure 4.2. PAP decreases activity of the capsaicin and noxious heat receptor TRPV1. (A) Capsaicin (1 μM)-evoked Ca^{2+} influx in Rat1 fibroblasts expressing TRPV1 alone or with TM-PAP. The 340/380 ratio is directly proportional to calcium concentration. n= 80 cells per condition. (B) Normalized capsaicin-evoked calcium responses in Rat1 fibroblasts transfected with the indicated constructs. The indicated cells were incubated with CPX (5 μM) for 3 hr prior to stimulation. n = 40-60 cells per condition. T tests relative to TRPV1 only condition. (C) The hindpaws of wild-type mice (WT, black) and *Trpv1*$^{-/-}$ mice (red) were tested for noxious thermal sensitivity before (baseline, BL) and after i.t. injection of S-hPAP (250 mU). Paired t tests were used to compare responses within each genotype to BL (black asterisks) and to compare responses between genotypes (red asterisks). n = 10 mice per genotype. (C-Inset) Detailed time course to determine onset of antinociception. (D) Mechanical (E) and thermal sensitivity of WT and *Trpv1*$^{-/-}$ mice before (BL) and after injection of Complete Freund's adjuvant (CFA) into one hindpaw. One day later, the

indicated mice were injected i.t. with S-hPAP (250 mU) or heat inactivated S-hPAP (0 mU). CFA-injected and non-injected (control) hindpaws were tested. Paired t tests were used to compare responses at each time point between WT mice and *Trpv1*$^{-/-}$ mice injected with S-hPAP (red asterisks). n = 8 mice per group. (F) Nocifensive licking responses to intraplantar (2 μg) and i.t. (0.5 μg) injection of capsaicin in WT and *Pap*$^{-/-}$ mice. n = 8 mice per group per condition. (B-F) **P* < 0.05, ***P* < 0.005, ****P* < 0.0005. All data are presented as means ± s.e.m. (Back to text)

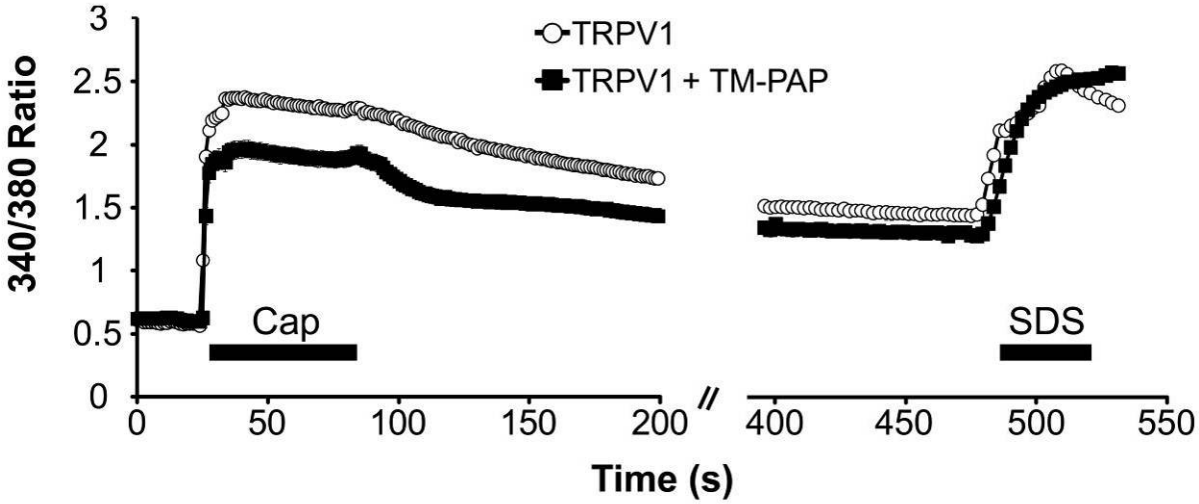

Figure 4.3. TM-PAP reduces capsaicin-evoked Ca^{2+} responses but not SDS-evoked Ca^{2+} responses. Raw (non-normalized) capsaicin (1 μM)- and SDS (0.06%)-evoked Ca^{2+} responses in Rat1 fibroblasts expressing TRPV1 alone or co-transfected with TM-PAP. Since TM-PAP did not affect SDS responses, SDS responses were used to normalize area under the curve (AUC) values in all calcium imaging experiments (see Experimental Procedures for details). n = 40 cells per condition. Points are mean 340/380 ratios ± s.e.m. Data up to the 200 s timepoint were also presented in Figure 1A. (Back to text)

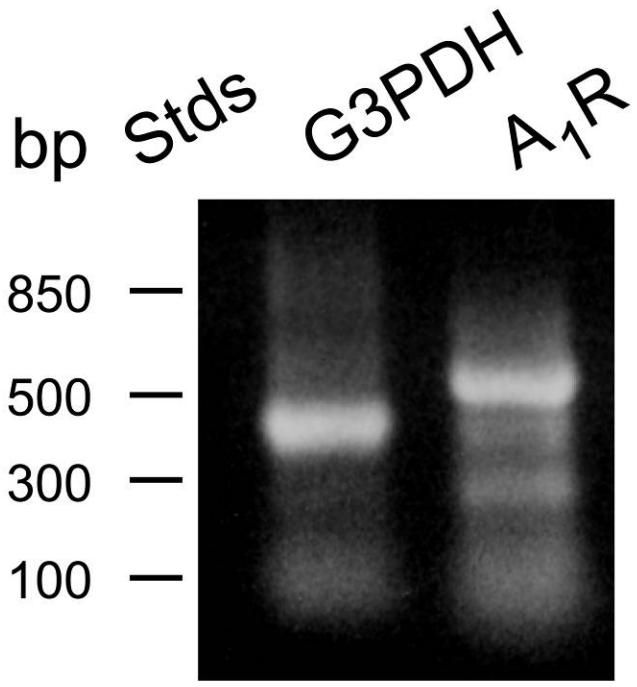

Figure 4.4. Rat1 fibroblasts express A_1R. RT-PCR on samples from Rat1 fibroblasts. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) positive control. (Back to text)

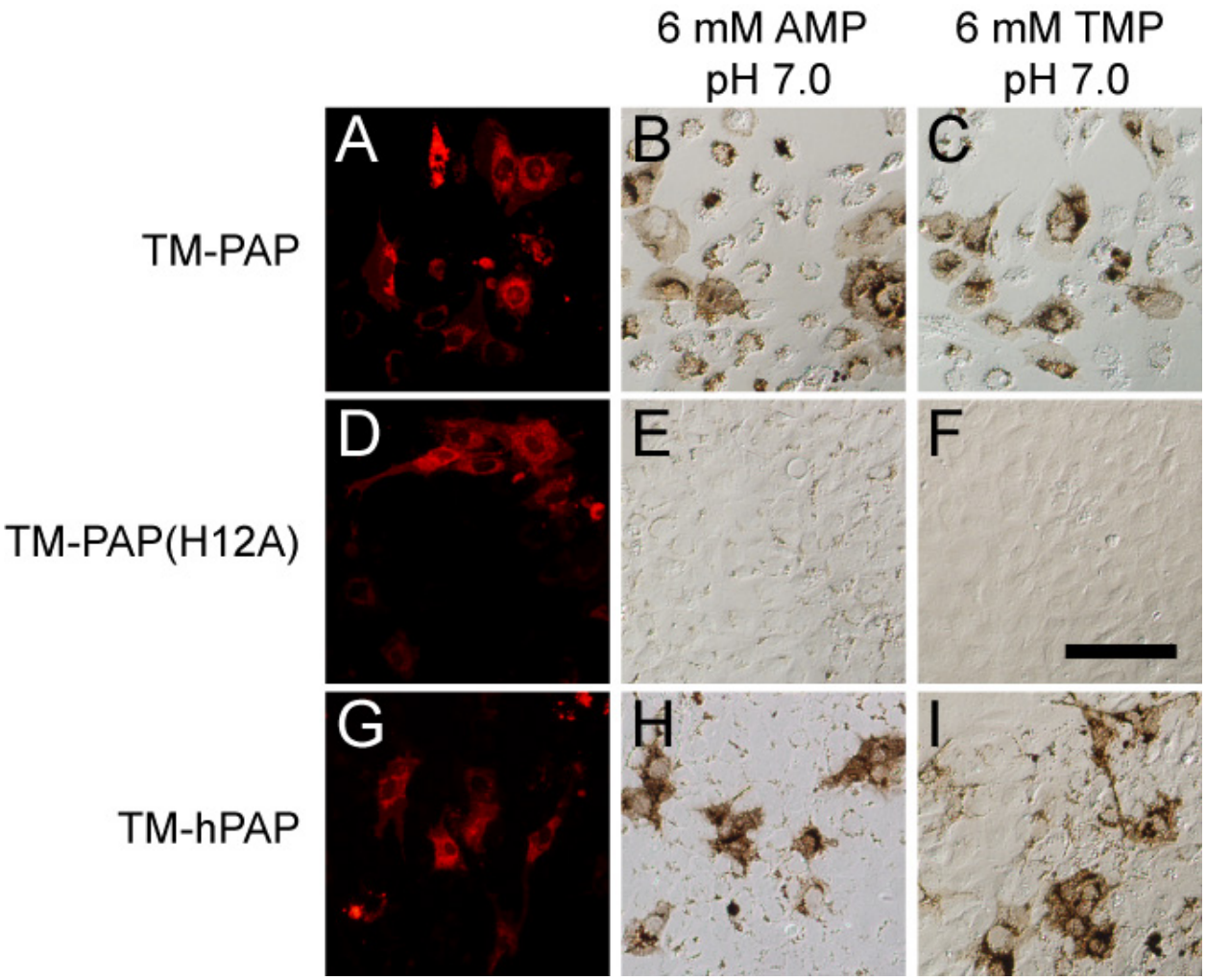

Figure 4.5. Mouse and human TM-PAP constructs dephosphorylate extracellular AMP and thiamine monophosphate (TMP) in Rat1 fibroblasts. Rat1 fibroblasts were transfected with (A-C) mouse TM-PAP, (D-F) mouse TM-PAP(H12A), or (G-I) human TM-PAP (TM-hPAP) and then imaged for (A, D, G) mCherry fluorescence or stained using (B, E, H) AMP or (C, F, I) TMP histochemistry at pH 7.0. The plasma membrane was not permeabilized, so that extracellular phosphatase activity could be assayed. AMP and TMP are PAP substrates. Scale bar is 50 μm. (Back to text)

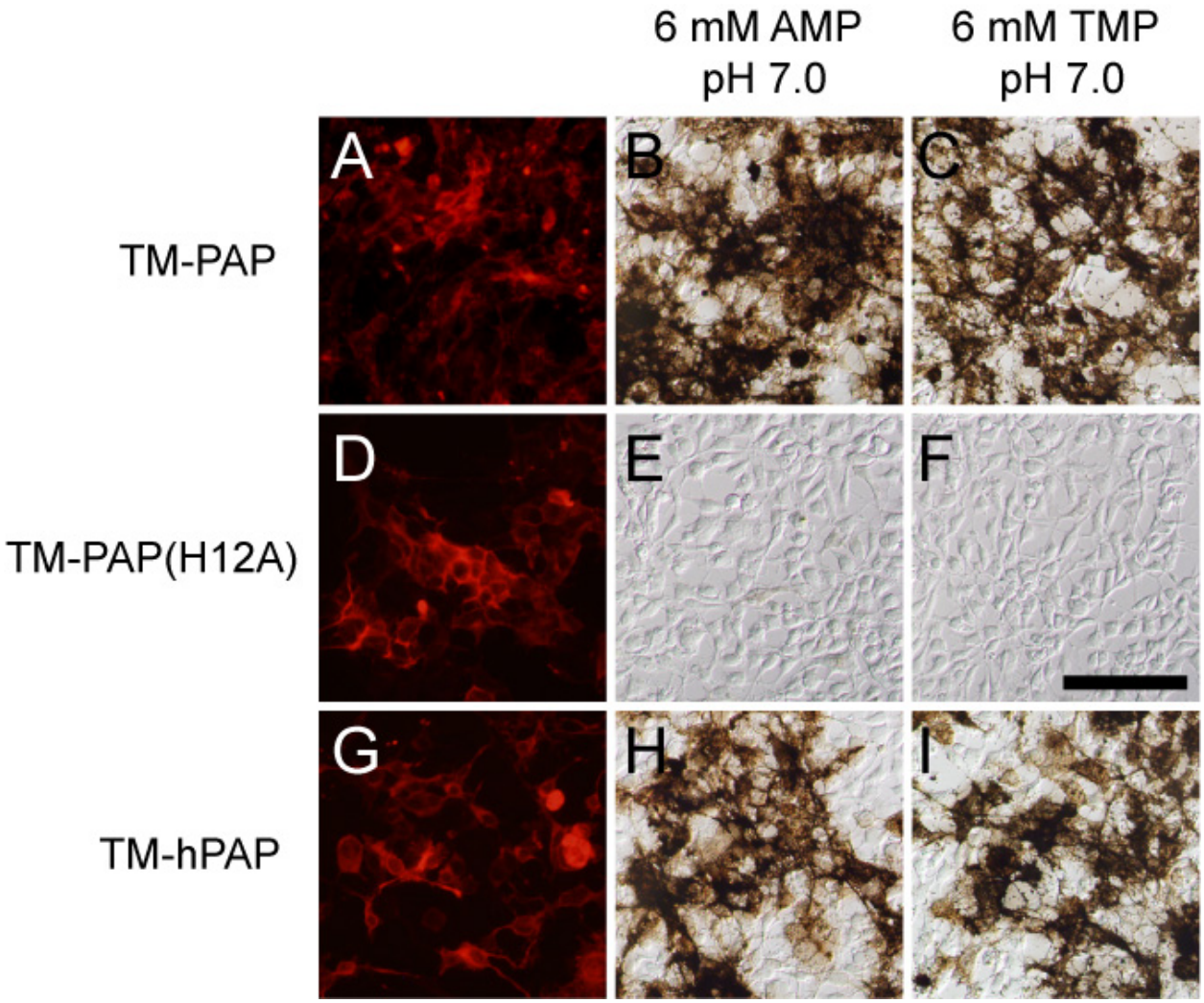

Figure 4.6. Mouse and human TM-PAP constructs dephosphorylate extracellular AMP and TMP in HEK293 cells. HEK293 cells were transfected with (A-C) mouse TM-PAP, (D-F) mouse TM-PAP(H12A), or (G-I) human TM-PAP (TM-hPAP) and then imaged for (A, D, G) mCherry fluorescence or stained using (B, E, H) AMP or (C, F, I) TMP histochemistry at pH 7.0. The plasma membrane was not permeabilized, so that extracellular phosphatase activity could be assayed. AMP and TMP are PAP substrates. Scale bar is 50 μm. (Back to text)

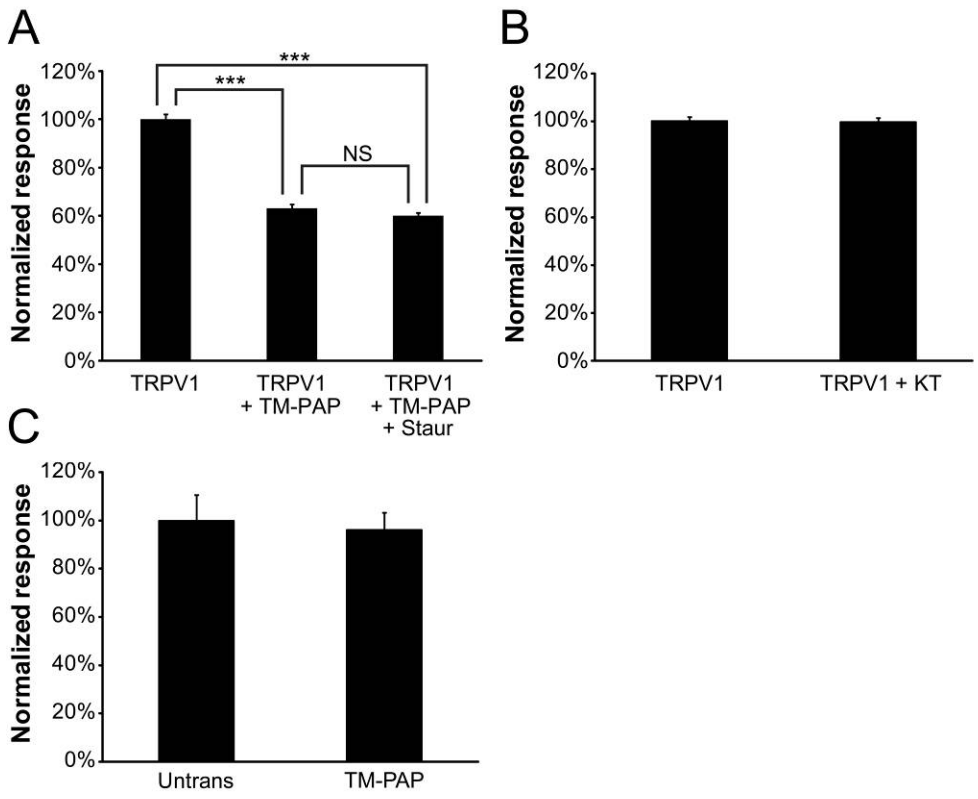

Figure 4.7. Activation of protein kinase C (PKC) or inhibition of protein kinase A (PKA) does not explain TM-PAP effect on capsaicin-evoked Ca^{2+} responses and TM-PAP does not deplete intracellular Ca^{2+} stores. (A) Normalized capsaicin (1 μM)-evoked Ca^{2+} responses in Rat1 fibroblasts expressing TRPV1 alone or co-transfected with TM-PAP. The indicated cells were incubated with the PKC inhibitor staurosporine (Staur, 100 nM) or (B) the PKA inhibitor KT5720 (KT, 1 μM) for 3 hr prior to stimulation. KT5720 did not mimic the effects of TM-PAP, ruling out the possibility that PAP reduced Ca^{2+} influx by $G\alpha_{i/o}$-mediated inhibition of PKA. (C) Normalized thapsigargin (TG, 10 mM)-evoked Ca^{2+} responses in untransfected Rat1 fibroblasts and in cells transfected with TM-PAP. Experiments were done in the presence of EGTA to reduce extracellular calcium. TG is an inhibitor of the endoplasmic reticulum calcium pump and evokes Ca^{2+} influx independent of IP_3. TM-PAP did not alter TG-evoked calcium responses, ruling out an effect of PAP on

calcium stores. n = 50-70 cells per condition. T tests relative to cells expressing TRPV1 alone. ***$P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

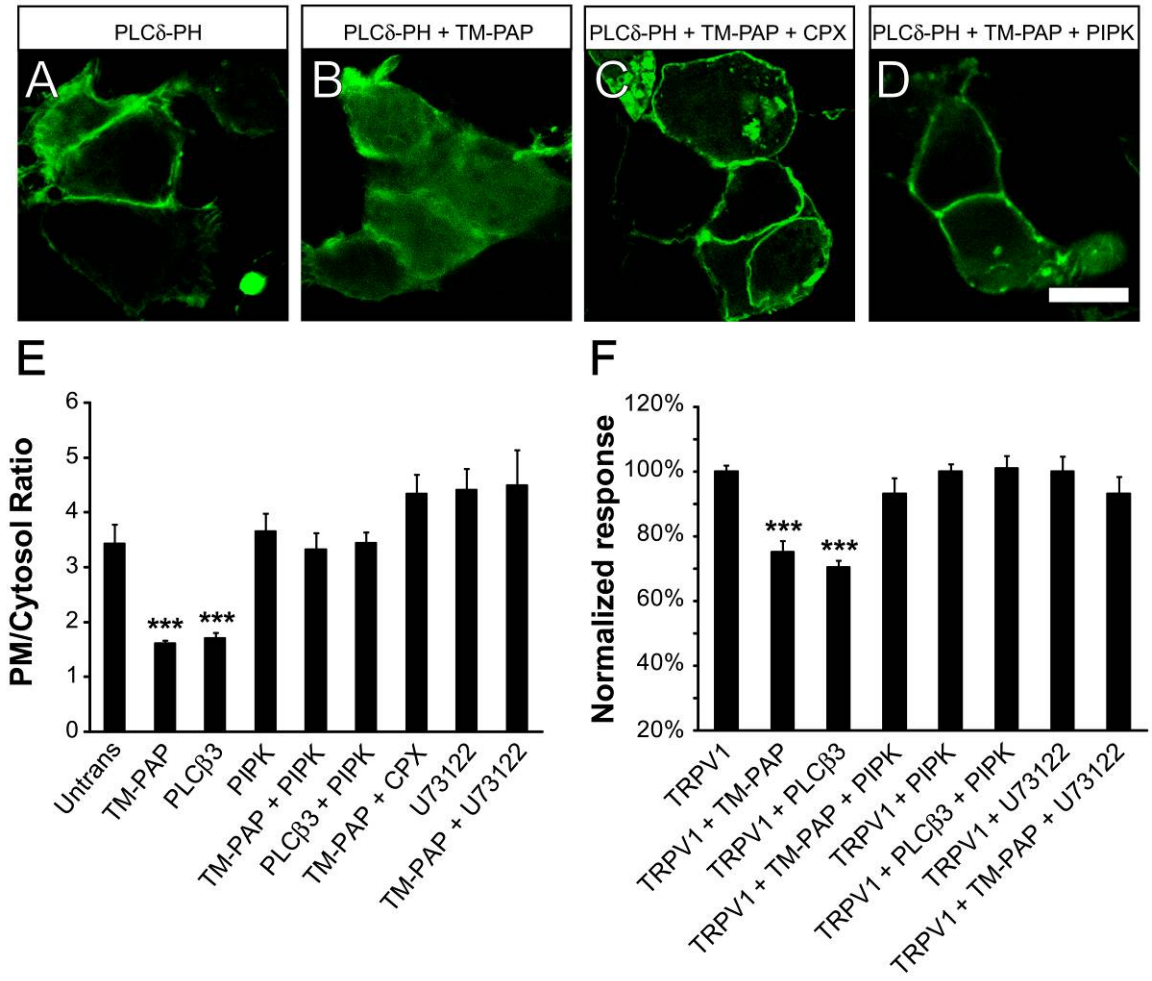

Figure 4.8. TM-PAP reduces capsaicin-evoked Ca^{2+} influx by decreasing PIP_2. (A-E) Subcellular localization of the PIP_2 biosensor PLCδ-PH-EGFP (PLCδ-PH) in HEK293 cells, imaged by confocal microscopy. PLCδ-PH (A) alone, (B) co-transfected with TM-PAP, (C) co-transfected with TM-PAP and incubated with 5 μM CPX for 3 hr prior to fixation, (D) co-transfected with TM-PAP and PIPK. Scale bar, 50 μm. (E) Quantification of PLCδ-PH subcellular localization after co-transfection with the indicated constructs or following incubation with CPX or U73122. Fluorescence values in the plasma membrane (PM) and cytosol were quantified from cell cross-sections using ImageJ and expressed as a ratio. n = 30-70 cells per condition. (F) Normalized capsaicin (1 μM)-evoked Ca^{2+} responses in Rat1

fibroblasts transfected with the indicated constructs. n = 40-100 cells per condition. T tests were used to compare (E) untransfected cells to transfected cells and (F) TRPV1 transfected cells to co-transfected cells. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

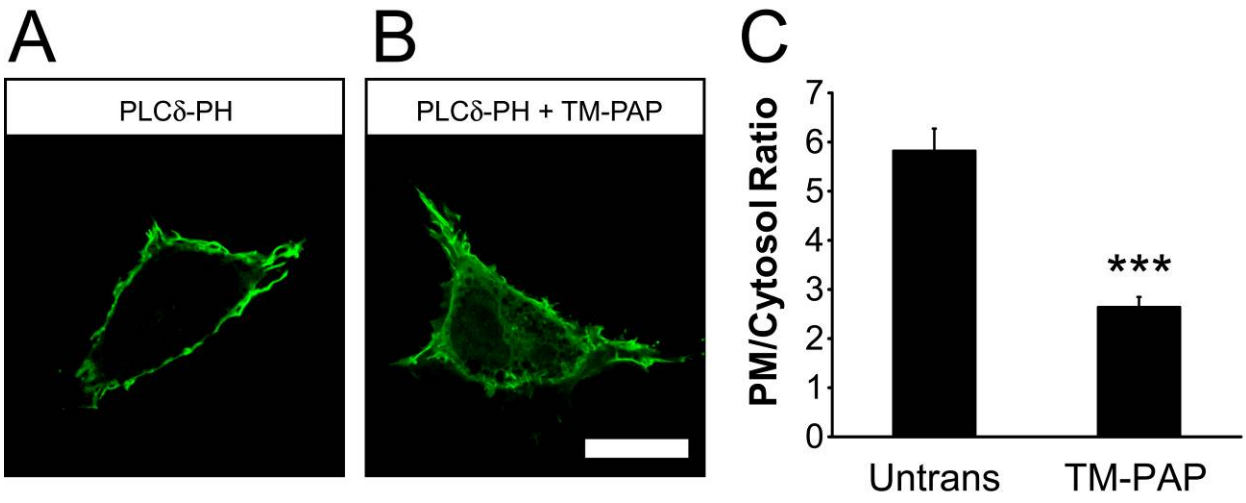

Figure 4.9. TM-PAP reduces PIP_2 levels in Rat1 fibroblasts. (A-C) Subcellular localization of PIP_2 biosensor PLCδ-PH-EGFP (PLCδ-PH) in transfected Rat1 fibroblasts, imaged by confocal microscopy. PLCδ-PH (A) was expressed alone or (B) with TM-PAP. Scale bar, 50 μm. (C) Quantification of PLCδ-PH subcellular localization alone or after co-transfection with TM-PAP. Fluorescence values in the plasma membrane (PM) and cytosol were quantified from cell cross-sections using ImageJ and expressed as a ratio. n = 20 cells per condition. T test was used to compare untransfected cells to TM-PAP transfected cells. ****P* < 0.0005. Data presented as means ± s.e.m. (Back to text)

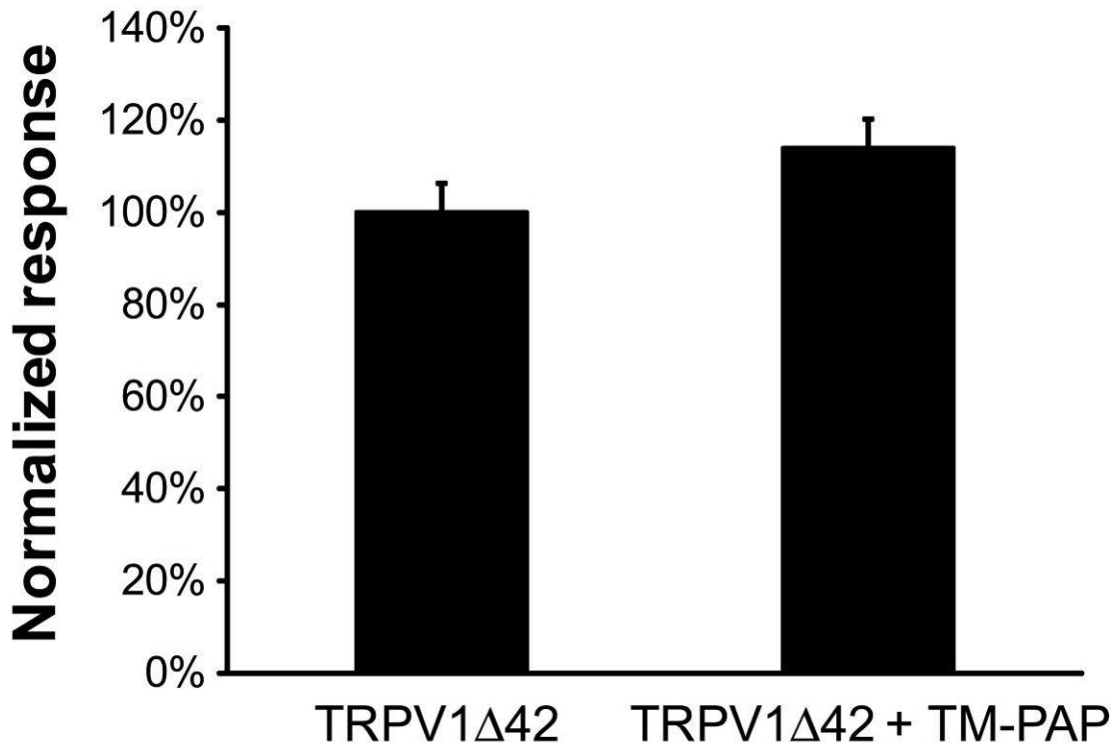

Figure 4.10. TM-PAP has no effect on capsaicin-evoked Ca^{2+} influx through TRPV1Δ42, a mutant version of TRPV1 that is missing a putative PIP_2-binding domain. Normalized capsaicin (1 μM)-evoked calcium responses in Rat1 fibroblasts transfected with the indicated constructs. n = 50-70 cells per condition. There were no significant differences between conditions. Values are presented as means ± s.e.m. (Back to text)

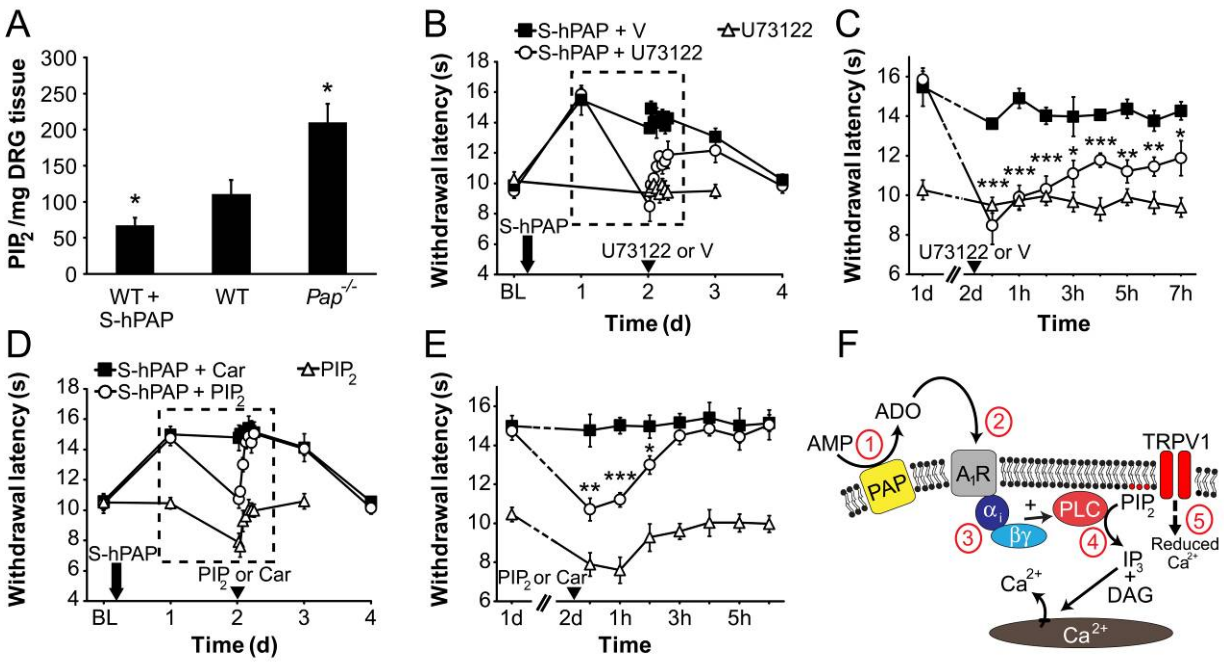

Figure 4.11. PAP activity alters PIP_2 levels in vivo. PIP_2 levels in L3-L6 DRG were quantified in wild-type (WT) mice, *Pap*$^{-/-}$ mice, or WT mice injected (i.t.) one day earlier with 250 mU S-hPAP. All mice were injected i.t. with lidocaine (see Experimental Procedures for details). n=3 mice per condition. (B, C) The hindpaws of wild-type mice were tested for noxious thermal sensitivity before (baseline, BL) and after i.t. injection of S-hPAP (250 mU) or saline. Two days later, the indicated mice were injected i.t. with either U73122 (5.4 nmol) or vehicle (V) and thermal sensitivity was measured every hour for the first 7 hours as well as for the next two days. Data in (C) is from the boxed area in (B). n = 8 mice per group. (D, E) The hindpaws of wild-type mice were tested for noxious thermal sensitivity before (BL) and after i.t. injection of S-hPAP (250 mU) or saline. Two days later, the indicated mice were injected i.t. with either PIP_2 (3 nmol) + carrier (Car) or carrier alone then thermal sensitivity was measured every hour for the first 6 hours after injection as well as for the next two days. Data in (E) is from the boxed area in (D). (F) Model showing how PAP interferes with TRPV1 channel activity (see text for details). For (A), values were

compared to WT by paired t test. For (C), mice injected with U73122 were compared to vehicle-injected mice at each time point by paired t test. For (E), mice injected with PIP_2 after S-hPAP were compared to vehicle-injected mice at each time point by paired t test. *P < 0.05, **P < 0.005, ***P < 0.0005. All data are presented as means ± s.e.m. (Back to text)

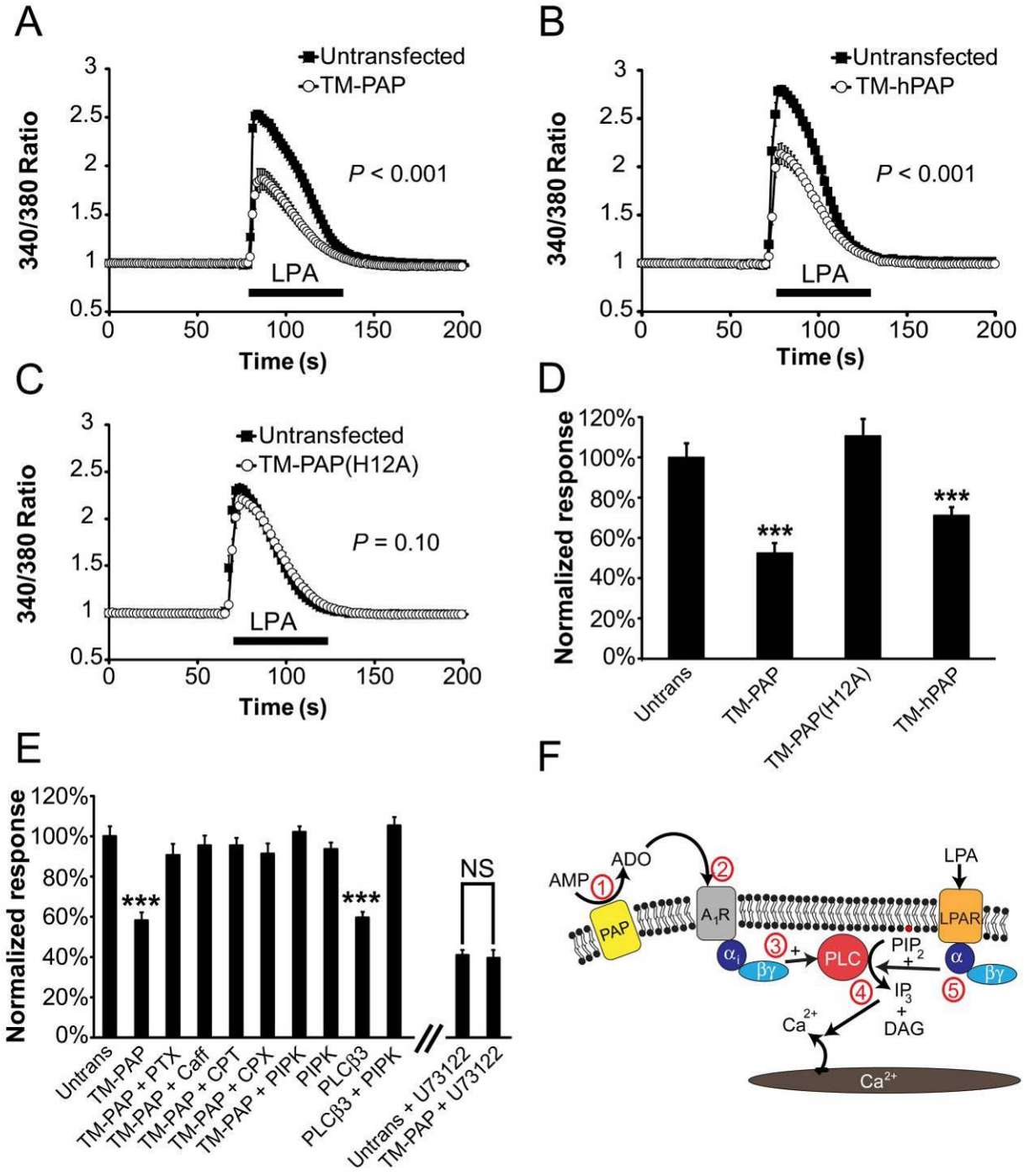

Figure 4.12. TM-PAP reduces LPA-evoked Ca^{2+} influx in Rat1 fibroblasts through activation of A_1R. (A-C) LPA (100 nM)-evoked Ca^{2+} influx in untransfected cells and in cells transfected with the indicated constructs. n=15 cells per condition. (D) Normalized LPA-evoked Ca^{2+}responses from four separate experiments. n = 40-60 cells per condition.

(E) LPA-evoked Ca^{2+} responses in cells expressing the indicated constructs and after incubation with PTX (500 ng/mL), caffeine (Caff, 1 mM), CPT (500 nM), CPX (5 μM) or U73122 (5 μM). Incubation time was 18 hours for PTX and 3 hours for all other compounds. Responses normalized to untransfected cells (Untrans). n = 70-100 cells per condition. (F) Model showing how PAP interferes with LPA receptor signaling (see text for details). For (A-C), 2-way ANOVA was used to compare transfected and untransfected cells (*P* values indicated on graphs). For (D, E), t tests were used to compare untransfected cells to transfected cells. ***$P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

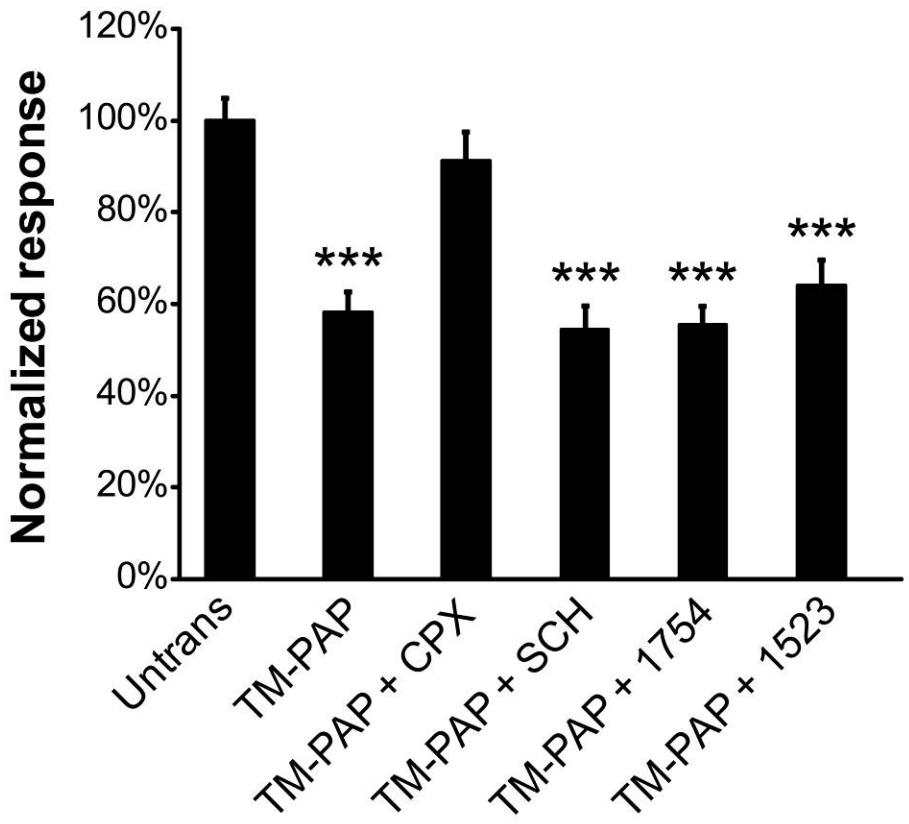

Figure 4.13. TM-PAP inhibits LPA-evoked Ca^{2+} responses via A_1R but not by other adenosine receptor subtypes. LPA-evoked Ca^{2+} responses in cells expressing mPAP-Cherry and after incubation with CPX (5 μM; A_1R antagonist), SCH58261 (500 nM; $A_{2A}R$ antagonist), MRS1754 (500 nM; $A_{2B}R$ antagonist), or MRS1523 (500 nM; A_3R antagonist). n = 75 cells per condition. T tests relative to untransfected cells. ****P* < 0.0005. All data are presented as means ± s.e.m. Untrans, TM-PAP, and TM-PAP + CPX data in this figure are the same as presented in Figure 4E. (Back to text)

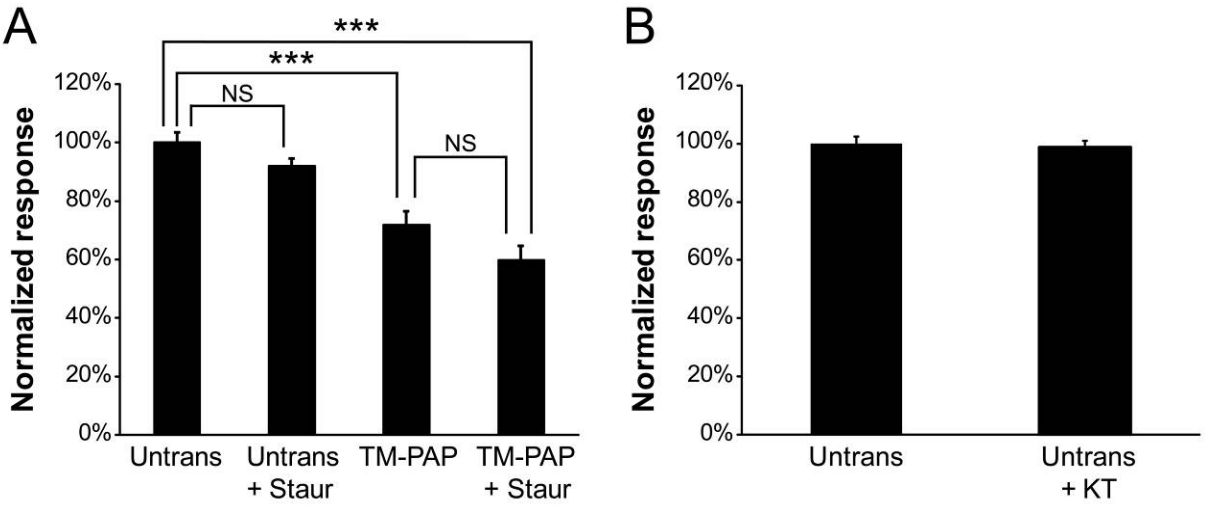

Figure 4.14. Activation of protein kinase C (PKC), inhibition of protein kinase A (PKA) or depletion of intracellular Ca^{2+} stores does not explain TM-PAP effect on LPA-evoked Ca^{2+} responses. (A) Normalized LPA (100 nM)-evoked Ca^{2+} responses in untransfected Rat1 fibroblasts and in cells transfected with TM-PAP. The indicated cells were incubated with the PKC inhibitor staurosporine (Staur, 100 nM) or (B) the PKA inhibitor KT5720 (KT, 1 μM) for 3 hr prior to stimulation. KT5720 did not mimic the effects of TM-PAP, ruling out the possibility that PAP reduces Ca^{2+} influx by $G\alpha_{i/o}$-mediated inhibition of PKA. n = 75 cells per condition. T tests relative to untransfected cells. ***$P <$ 0.0005. (Back to text)

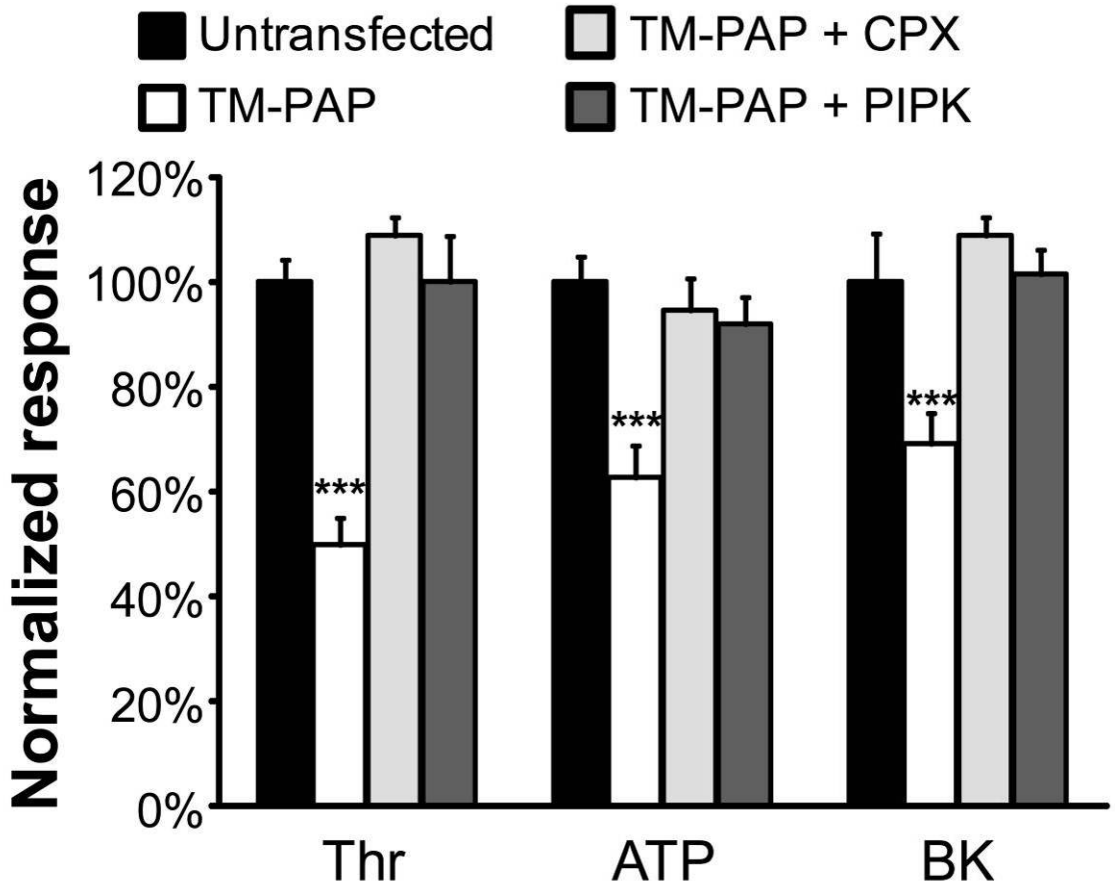

Figure 4.15. TM-PAP reduces signaling through diverse pro-nociceptive receptors in an A_1R-dependent manner. Normalized Ca^{2+} responses in untransfected Rat1 fibroblasts or in cells transfected with the indicated constructs and stimulated with LPA (100 nM), thrombin (Thr, 1 U/mL), ATP (10 μM) or bradykinin (BK, 1 μM). Some cells were incubated with CPX (5 μM) for 3 hr prior to stimulation. Responses were normalized to untransfected cells stimulated with these ligands. n = 70-110 cells per condition. T tests relative to untransfected cells. ***$P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

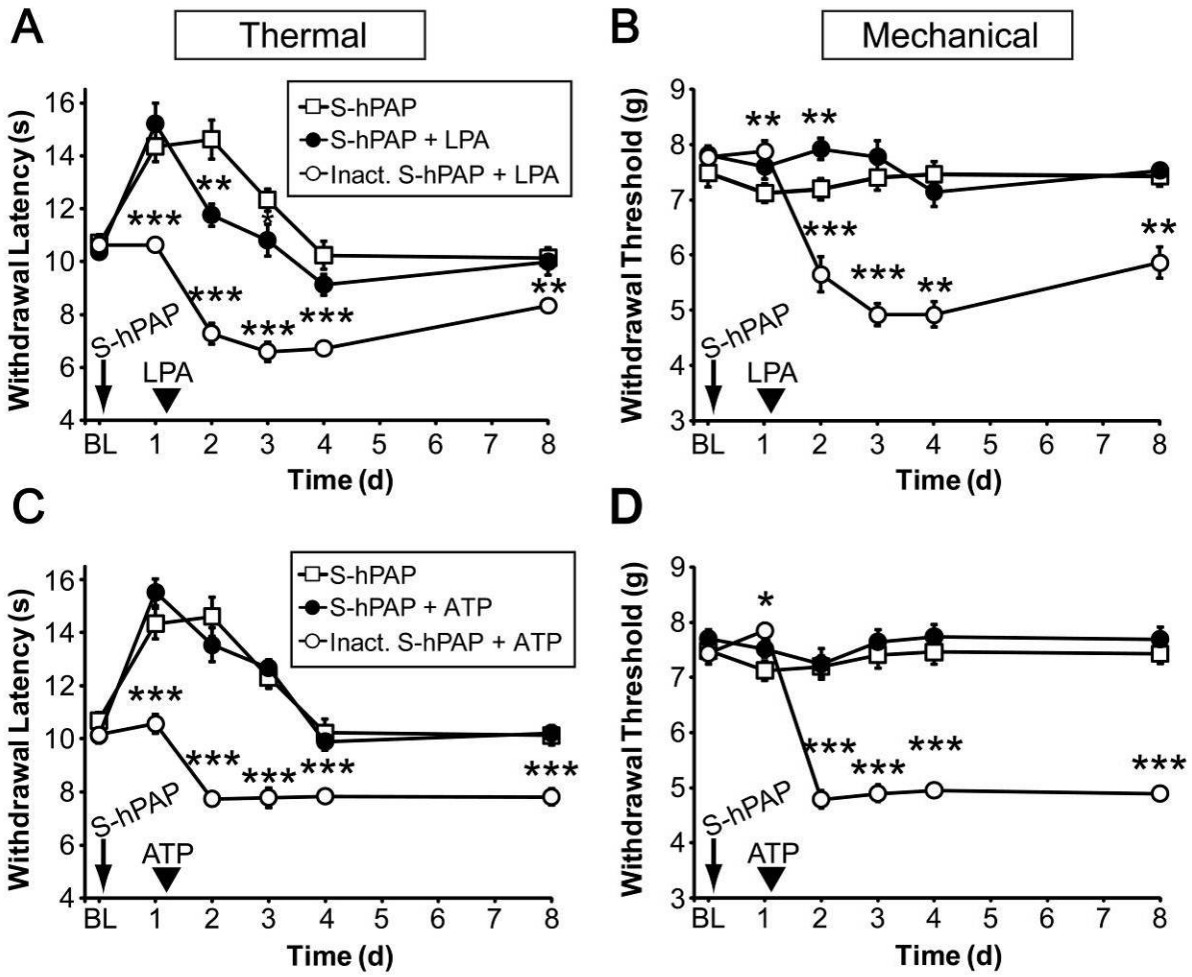

Figure 4.16. Secretory PAP inhibits pro-nociceptive receptor signaling in vivo. (A-D) The hindpaws of wild-type mice (n=10 per group) were tested for noxious thermal and mechanical sensitivity before (baseline, BL) and after i.t. injection of S-hPAP (250 mU) or heat inactivated S-hPAP (0 mU). One day later, the indicated mice were injected i.t. with (A, B) 5 nmol LPA or (C, D) 100 nmol ATP. Paired t tests were used to compare responses at each time point (A-D) between mice injected with active S-hPAP (open squares) to mice in the two other groups. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

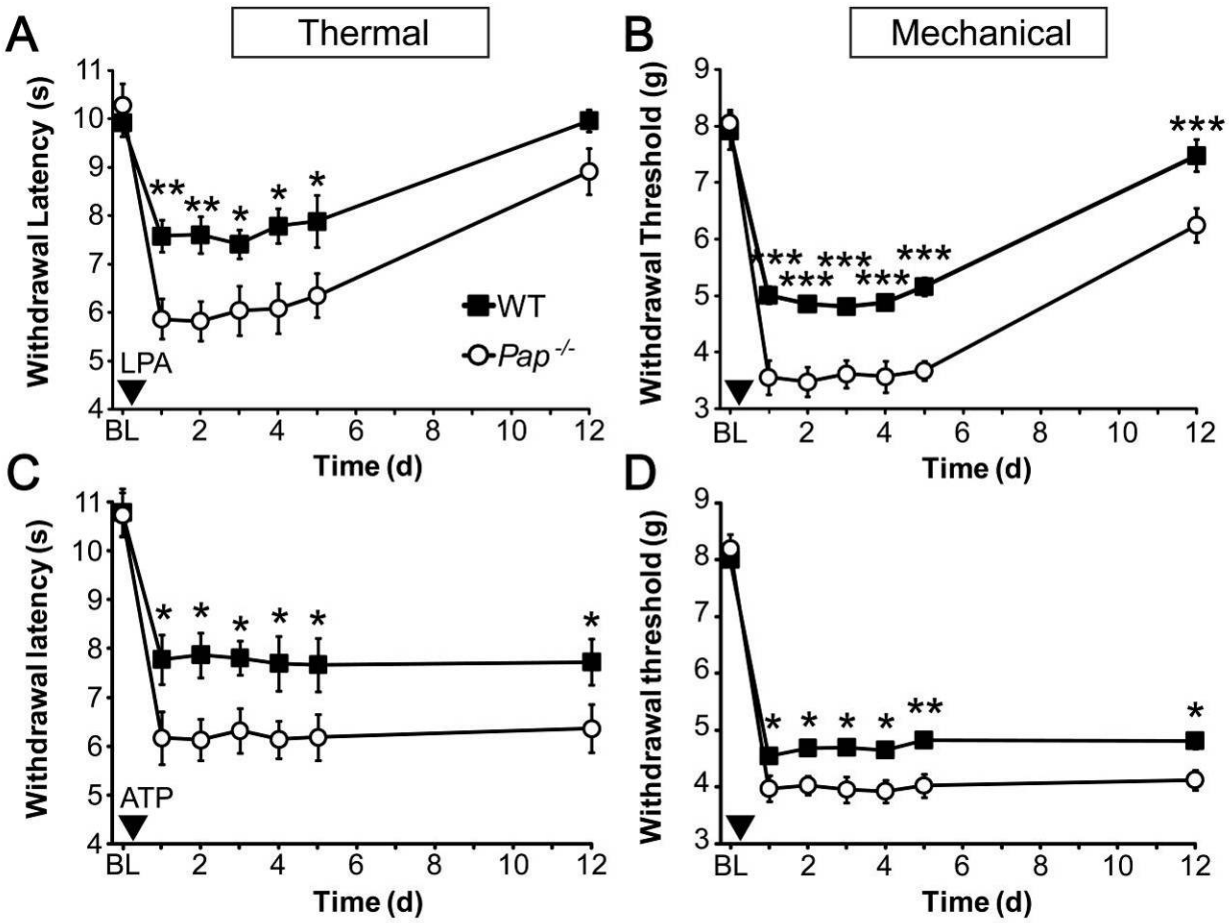

Figure 4.17. *Pap*$^{-/-}$ mice show enhanced LPA- and ATP-induced thermal hyperalgesia and mechanical allodynia. (A, B) The hindpaws of wild-type (WT) and *Pap*$^{-/-}$ mice were tested for (A) noxious thermal and (B) mechanical sensitivity before (baseline, BL) and after i.t. injection of LPA (5 nmol) or ATP (100 nmol). n = 10 mice per genotype. *Pap*$^{-/-}$ mice developed significantly greater thermal hyperalgesia ($P < 0.0001$ by 2-way ANOVA) and mechanical allodynia ($P < 0.0001$ by 2-way ANOVA) in response to i.t. LPA or ATP. Post-hoc paired t tests were used to compare responses at each time point between genotypes. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

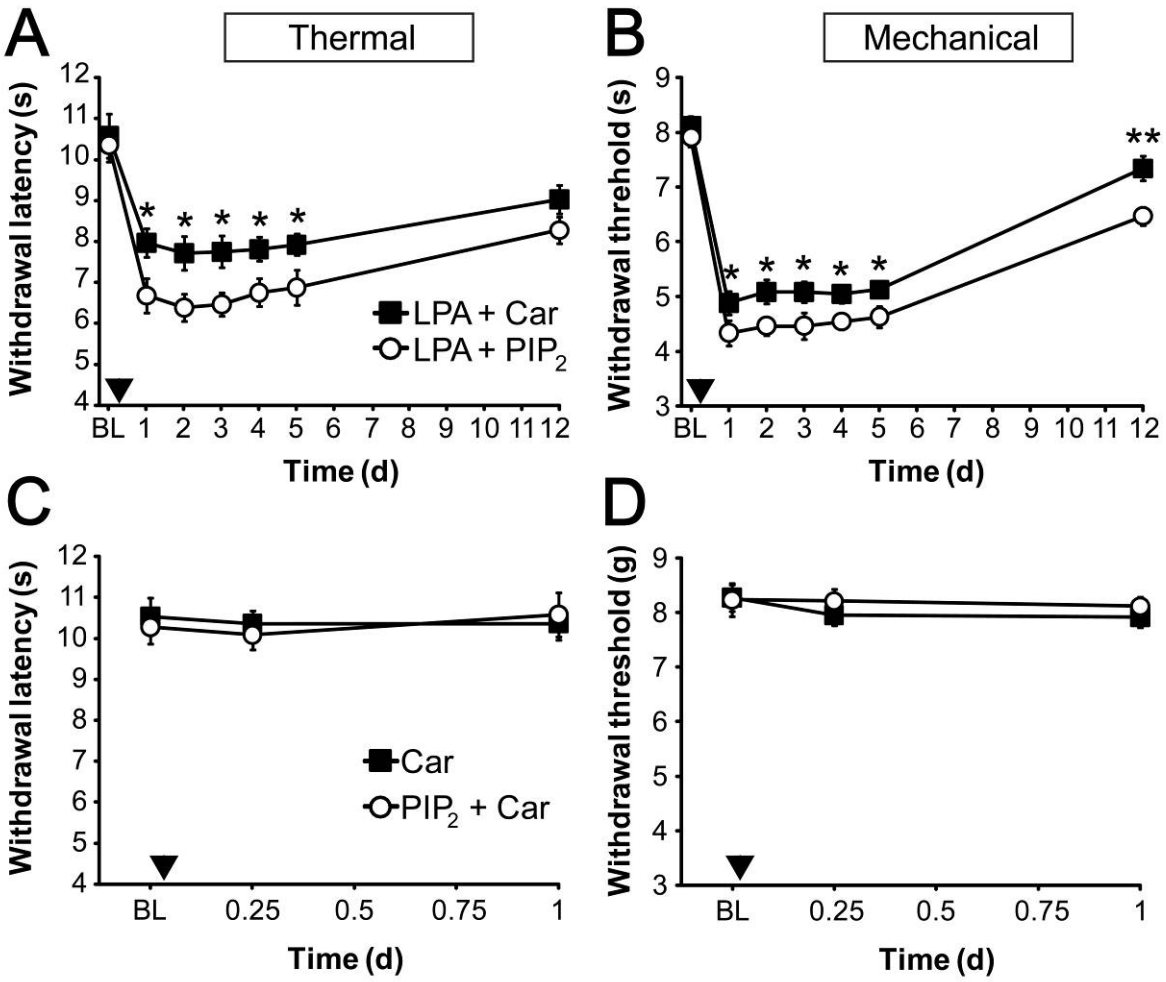

Figure 4.18. Co-injection with PIP_2 enhances LPA-induced hyperalgesia and allodynia. (A, B) The hindpaws of wild-type mice were tested for (A) noxious thermal and (B) mechanical sensitivity at baseline (BL). The same day, the mice were injected i.t. with either LPA (5 nmol) + PIP_2 (3 nmol) + carrier (Car) or LPA + Car alone, and thermal sensitivity was measured on the indicated days. (C, D) The hindpaws of wild-type mice were tested for (C) noxious thermal and (D) mechanical sensitivity at baseline (BL). The same day, the indicated mice were injected i.t. with either PIP_2 (3 nmol) + carrier (Car) or carrier alone, and thermal sensitivity was measured. Paired t tests were used to compare groups at each time

point. *P < 0.05, **P < 0.005, ***P < 0.0005. All data are presented as means ± s.e.m.

(Back to text)

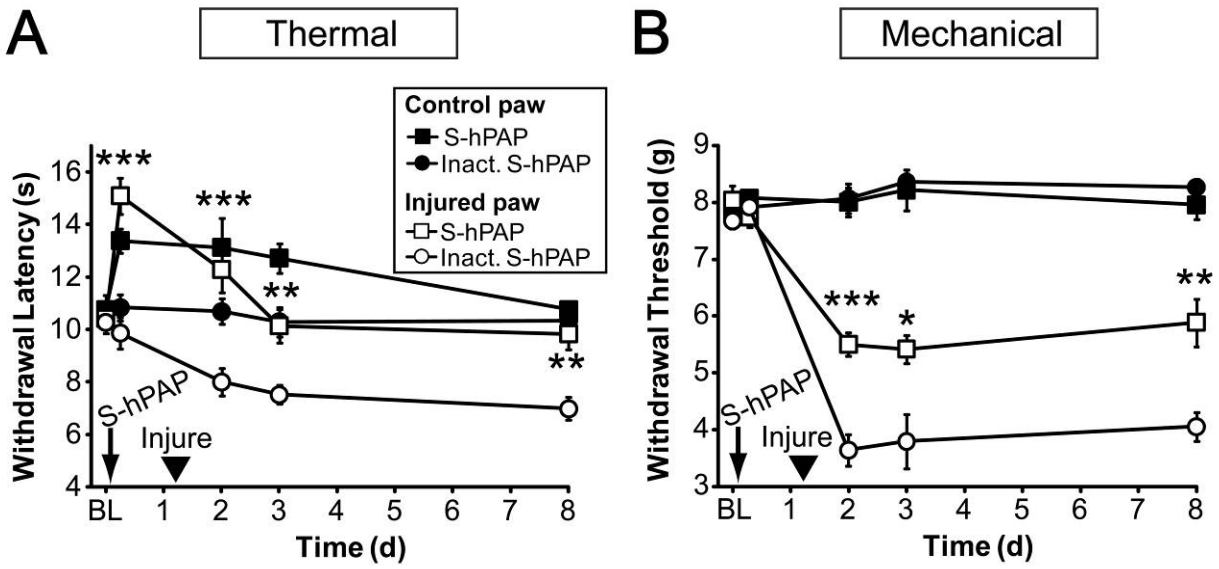

Figure 4.19. Secretory PAP partially blocks the initiation of neuropathic pain. (A, B) The hindpaws of wild-type mice (n=10 per group) were tested for noxious thermal and mechanical sensitivity before (baseline, BL) and after i.t. injection of S-hPAP (250 mU) or heat inactivated S-hPAP (0 mU). One day later, peripheral nerves were injured using the SNI model of neuropathic pain (Injure-arrowhead). Injured and non-injured (control) hindpaws were tested. Paired t tests were used to compare responses at each time point between mice injected with S-hPAP (open square; injured paw) and those injected with inactive hPAP (open circles; injured paw). $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

Table 4.1. No significant differences in the percentage of TRPV1$^+$ L4-L6 DRG neurons in WT verses *Pap*$^{-/-}$ mice

	TRPV1$^+$ DRG neurons	NeuN$^+$ DRG neurons	% TRPV1$^+$
Wild-type	1120	3141	35.7 ± 1.7
Pap$^{-/-}$	1340	4051	33.1 ± 0.7

$P = 0.115$

Percentage expressed as mean ± s.e.m. DRG from three mice per genotype were immunostained and counted (total of 12 DRG sections per genotype). Counter was blind to genotype.
(Back to text)

CHAPTER 5

Ecto-5'-nucleotidase is Present in Nociceptive Neurons and Inhibits Pain Transmission

ABSTRACT: Ecto-5'-nucleotidase (NT5E) is a 5'-AMPase present in many tissues. It rapidly converts 5'AMP to adenosine, affecting numerous physiologic processes. We have recently shown that another ectonucleotidase, prostatic acid phosphatase (PAP), is present in nociceptive (pain-sensing) neurons where it plays an important role in the modulation of nociceptive signaling. Previous studies hinted that NT5E is present and functional in nociceptive circuits, but definitive studies are lacking. Here we show, using *Nt5e* knockout mice, that NT5E is expressed in nociceptive neurons in the DRG that terminate in laminae I and II of the dorsal spinal cord. NT5E can degrade AMP to adenosine in these regions in situ and in vivo. Loss of NT5E leads to increased thermal hyperalgesia and mechanical allodynia following inflammatory insult or peripheral nerve injury. Conversely, injection of NT5E protein has antinociceptive, antihyperalgesic, and antiallodynic effects that depend on activation of A_1-adenosine receptors. These properties were similar to that seen following injection of PAP. These studies reveal an important role for NT5E in the modulation of nociception, as well as highlight the potential of ectonucleotidase-targeted therapies for the treatment of chronic pain.

This work is in preparation for submission: Sowa, N.A., Taylor-Blake, B., Voss, M., and Zylka, MJ. *Manuscript in preparation*.

5.1) Introduction

Nucleotides play important roles in the initiation and maintenance of pain (Burnstock, 2007; Sawynok, 2007; Tozaki-Saitoh et al., 2008; Tsuda et al., 2005). Nucleotides like ATP and ADP are released extracellularly from stimulated sensory neurons and activate purinergic P2X and P2Y receptors on neurons and microglia. Activation of these receptors facilitates neurotransmission, sensitizes neurons, and causes or enhances pain. The effects of ATP and ADP are terminated through the action of ectonucleotidases. These enzymes degrade ATP, ADP, and AMP in a step-wise manner to adenosine, which either acts on its receptors or is taken up into cells and converted back to AMP or into inosine (Sawynok and Liu, 2003; Zimmermann, 2006). Activation of central adenosine receptors inhibits neuronal transmission and suppresses pain (Nakagawa et al., 2007; Sawynok, 2007). Thus, ectonucleotidases can suppress pain in two ways: 1) through removal of pro-nociceptive ATP and ADP, and 2) through production of antinociceptive adenosine.

Ectonucleotidases have been putatively studied in nociceptive circuits for almost 50 years (Nagy and Daddona, 1985; Scott, 1965; Scott, 1967; Suran, 1974). Their presence was determined through the use of enzyme histochemical techniques that rely on the degradation of adenine-containing nucleotides in situ. However, these techniques are not specific for a given enzyme, as several enzymes are capable of performing these reactions. Thus, despite their potential importance in the modulation of pain, the exact molecular identity of the ectonucleotidases present in nociceptive circuits remains largely unknown.

Recently, we identified prostatic acid phosphatase (PAP) as the first ectonucleotidase present in nociceptive circuits (Zylka et al., 2008). PAP is found predominantly in nonpeptidergic nociceptive neurons in the dorsal root ganglia (DRG) whose axons terminate

in lamina II of the dorsal horn of the spinal cord. Genetic deletion of PAP leads to enhanced nociceptive responses in models of inflammatory and neuropathic pain. Further, injection of a soluble form of the PAP protein inhibits nociception in several pain models. These effects are due to PAP's ability to degrade AMP to adenosine and activate anti-nociceptive A_1-adenosine receptors (A_1Rs). These studies not only identified PAP as the first known ectonucleotidase in nociceptive circuits, but also confirmed the importance of ectonucleotidases in general in the modulation of pain. Interestingly, mice null for PAP (*Pap*$^{-/-}$) retain some residual AMPase activity in the DRG and dorsal spinal cord. This led us to investigate the identity of additional ectonucleotidases present in these regions which may also be involved in nociceptive signaling.

Ecto-5'-nucleotidase (NT5E) is a nucleoside 5'-monophosphatase that dephosphorylates AMP to adenosine in many different tissues. Through this action, NT5E plays an important role in epithelial fluid transport, maintenance of tissue barrier function, adaptation to hypoxia and ischemia, and inflammation (Colgan et al., 2006). NT5E has been thought to be expressed in nociceptive pathways based on enzyme histochemical studies using AMP as a substrate (Nagy and Daddona, 1985; Scott, 1967; Suran, 1974). Recent studies also suggest a possible functional role for NT5E in the modulation of nociceptive signaling, based on the measurement of AMPase activity in the spinal cord (Fontella et al., 2005; Patterson et al., 2001). However, other enzymes, such as PAP, are also capable of degrading AMP to adenosine, and thus, definitive proof for the expression and function of NT5E in nociceptive circuits is lacking.

Here, we used immunofluorescence in wild-type mice and mice null for NT5E (*Nt5e*$^{-/-}$), to definitively show that NT5E is expressed in nociceptive circuits and can degrade AMP

to adenosine in these regions. Further, we showed an important functional role of NT5E in nociception, as loss of NT5E led to increased thermal hyperalgesia and mechanical allodynia following inflammation or nerve injury. Conversely, addition of NT5E, through intraspinal injection of recombinant NT5E protein, had antinociceptive, antihyperalgesic, and antiallodynic effects that lasted for up to 2 days. These antinociceptive properties required the presence of A_1R, confirming that NT5E converts AMP to adenosine in vivo. Together our results not only identify NT5E as one of the major AMPases in nociceptive circuits, but also emphasize the importance of ectonucleotidases in the modulation of nociception and their viability as a potential target for the treatment of chronic pain.

5.2) Materials and Methods

All procedures and behavioral experiments involving vertebrate animals were approved by Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

5.2.1) Molecular Biology and Protein Purification

The mNT5E-His6 baculovirus expression clone (encompassing nt 130 -1696 from GenBank accession # NM_011851) was generated by PCR amplification, using a full-length expression construct of mNT5E as template and Phusion polymerase. PCR products were cloned into pAcSecG2T (BD Biosciences) and completely sequenced. Primer sequences contained EcoRI sites (underlined) to facilitate cloning (N-terminal primer: 5'-cgcgaattcattgggagctcacgatcctgcacaca. C-terminal –His6 tag primer: 5'-gcggaattcttaatgatgatgatgatgatggaacttgatccgcccttcaacg). This vector was used to generate recombinant mNT5E protein using the Bac-to-Bac Baculovirus Expression System

(Invitrogen). Briefly, we infected Hi5 insect cells with high-titer recombinant baculovirus, incubated the cells for 48 hours at 27°C, then harvested and concentrated the supernatant containing secreted mNT5E protein. Then, mNT5E protein was purified from the concentrated supernatant using Ni-NTA HisTrap agarose (GE Healthcare Life Sciences) affinity chromatography and imidazole as eluant. Lastly, mNT5E protein was dialyzed against PBS to remove imidazole. Protein purity was confirmed by SDS-PAGE, staining for total protein with GelCode Blue (Pierce/Thermo Scientific, Cat. # 24590) and western blotting with anti-mNT5E antibody (RD Systems, Cat. # AF4488). Recombinant mNT5E was kept at 4°C for short-term (1-2 months) use and at -80°C for long-term storage.

5.2.2) Tissue Preparation

Adult male mice, 6–12 weeks of age, were sacrificed by cervical dislocation, decapitation, or pentobarbital overdose. Lumbar spinal cord and DRG (L4–L6) were dissected and then immersion fixed for 8 hr and 2 hr, respectively, in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4. Tissues were cryoprotected in 20% sucrose, 0.1 M phosphate buffer, pH 7.3 at 4°C for 24 hr, frozen in OCT, sectioned with a cryostat at 15–20 μm, and mounted on Superfrost Plus slides. Slides were stored at −20°C. Free-floating sections were sectioned at 30 μm and immediately stained.

For DRG cultures, all DRGs were collected and pooled from adult male wild-type and *Nt5e*$^{-/-}$ mice. DRGs were dissociated using collagenase (1 mg/mL; Worthington, CLS1) + dispase (5 mg/mL; Gibco, 17105-041) in HBSS and neurons were cultured on poly-D-lysine + laminin-coated glass coverslips in DH10 media (1:1 Hams F12/DMEM + 10 % FBS

+ 1 % penicillin/streptomycin) with 25 ng/mL GDNF (Chemicon, GF030) at 37C. Histochemistry was performed 72 hours after culturing.

5.2.3) Histology

Enzyme histochemistry was performed essentially as described previously (Zylka et al., 2008) using 3 mM or 6 mM AMP as substrate and Tris-maleate buffer at pH 5.6 or 7.0. Immunofluorescence was performed essentially as described (Zylka et al., 2005), although we substituted high salt TBS + TX (50 mM Tris, 2.7% NaCl, 0.3% Triton X-100, pH 7.6) for PBS + TX in all wash and antibody incubation steps. Additional antibodies included 1:50 (DRG) or 1:100 (spinal cord) sheep anti-NT5E (AF4488, R+D Systems), 1:750 rabbit anti-CGRP (T-4032; Peninsula**),** 1:250 mouse anti-NeuN (MAB377, Chemicon)**,** 1:750 rabbit anti-$P2X_3$ (RA10109, Neuromics)**,** 1:750 rabbit anti-TRPV1 (RA14113, Neuromics)**,** 1:600 (DRG) or 1:750 (spinal cord) chicken anti-GFP (GFP-1020, Aves), 1:500 rabbit anti-NF200 (AB1982, Chemicon), and 1:4000 chicken anti-human PAP (in-house), with amplification as described previously (Zylka et al., 2008)**.** Secondary antibodies used included 1:25 rat anti-mouse IgG1-FITC (Zymed/Invitrogen), 1:200 donkey anti-chicken IgY-Biotin (Jackson), 1:200 donkey anti-chicken IgY-FITC (Jackson). IB4 (Invitrogen) was used at 1:100. Images were obtained using a Leica TCS-NT confocal microscope.

5.2.4) Intrathecal Injections

We used concentrated mNT5E protein (0.34 units/μL; 0.48 μg/μL) for injections. mNT5E activity was determined by malachite green assay using AMP as substrate at pH 7.0

as described previously (Sowa et al., 2009). For injections of AMP and ITU, 5'-adenosine monophosphate (Fluka, 01930) and 5-iodotubericidin (Biomol, EI-293) were made up in 0.9% saline and DMSO stock solutions and diluted to final concentrations in 0.9% saline. All solutions were intrathecally injected (5 μL) into unanesthetized mice using the direct lumbar puncture method (Fairbanks, 2003).

5.2.5) PIP_2 Quantification

For quantification of PIP_2 in dorsal root ganglia (DRG), age-matched, adult male C57BL/6, $A_1R^{-/-}$, or $Nt5e^{-/-}$ mice were sacrificed and L3-L6 DRGs were dissected bilaterally (n=8 ganglia / sample) and placed in PBS on ice. For each sample, DRG wet weight was determined and then lipids were extracted and quantified using the $PI(4,5)P_2$ Mass ELISA Kit from Echelon (K-4500) following the manufacturer's protocol. PIP_2 levels were normalized by dividing by the wet weight of the DRG tissue.

5.2.6) Behavior

$Nt5e^{-/-}$ and $A_1R^{-/-}$ mice were backcrossed to C57BL/6 mice (Jackson) for 10 and 12 generations, respectively. Isogenic wild-type mice were then derived from the $Nt5e^{-/-}$ line and used as wild-type controls. C57BL/6 male mice were purchased from Jackson Laboratories for all behavioral experiments involving mNT5E protein injections. Unless indicated otherwise, male mice, 2–4 months old, were used for all behavioral experiments. All mice were acclimated to the testing room, equipment, and experimenter for 1–3 days before behavioral testing. The experimenter was blind to genotype and protein injection during behavioral testing.

Thermal sensitivity was measured by heating one hindpaw with a Plantar Test apparatus (IITC) following the Hargreaves method (Hargreaves et al., 1988). The radiant heat source intensity was calibrated so that a paw-withdrawal reflex was evoked in ~ 10 s., on average, in wild-type C57BL/6 mice. Cutoff time was 20 s. One measurement was taken from each paw per day to determine paw-withdrawal latency, with the exception of the AMP ± ITU experiments, where measurements were made every hour for 6 hours after injection. To perform the tail-immersion assay, mice were gently restrained in a towel and the distal one-third of the tail was immersed in 46.5°C or 49°C water. Latency to withdrawal the tail was measured once per mouse. For the hot plate test, mice were placed on a metal surface heated at 52°C or 55ºC and latency to jump or lick the hindpaws was measured. Mechanical sensitivity was measured using semiflexible tips attached to an electronic von Frey apparatus (IITC) as described elsewhere (Cunha et al., 2004; Inoue et al., 2004). Three measurements were taken from each paw (separated at 10 min intervals) then averaged to determine paw-withdrawal threshold in grams.

To induce inflammatory pain, 20 μl complete Freund's adjuvant (CFA, from Sigma or MP Biomedicals) was injected into one hindpaw, centrally beneath glabrous skin, with a 30G needle. The spared nerve injury (SNI) model of neuropathic pain was performed as described (Shields et al., 2003).

5.3) Results

5.3.1) NT5E is Expressed in DRG Sensory Neurons and Produces Adenosine

NT5E is thought to be expressed in mouse DRG and spinal cord based on histochemical assays that utilize the degradation of nucleotide monophosphates to stain these

tissues. However, other enzymes present in these regions, such as PAP, are also capable of degrading nucleotide monophosphates (Zylka et al., 2008). It is thus difficult to conclude based solely on functional assays if NT5E is indeed expressed in DRG and spinal cord. To test if NT5E protein is expressed in these regions, we performed double-label immunofluorescence with a commercially available anti-NT5E antibody and antibodies for various sensory neuron markers in these tissues. The anti-NT5E antibody specificity was confirmed by both an absence of staining when primary antibody was excluded and an absence of staining in tissues from *Nt5e*$^{-/-}$ mice (data not shown) (Thompson et al., 2004a).

In agreement with published studies using histochemical staining, NT5E is predominantly expressed in small diameter DRG neurons, with only a small percentage of large-diameter neurons showing NT5E staining (Figures 5.1A-5.1V). Cell counts from confocal images revealed that only 4.5 ± 0.9% of NT5E^{+} neurons express NF200, a marker of myelinated neurons (Figures 5.1T-5.1V and Table 5.1). NT5E is expressed in the vast majority of nonpeptidergic DRG neurons, as defined by the markers IB4, *Mrgprd-EDGPf*, and $P2X_3$ (Figures 5.1D-5.1L and Table 5.1). Interestingly, nearly all NT5E^{+} neurons also express PAP, which is also predominantly found in nonpeptidergic nociceptors (Figures 5.1A-5.1C and Table 5.1) (Zylka et al., 2008). In addition, NT5E was also expressed in a substantial number (38.0 ±2.4%) of peptidergic CGRP^{+} neurons (Figures 5.1M-5.1O and Table 5.1). Fittingly, NT5E^{+} axons terminated in both laminae I and II in the spinal cord in association with peptidergic and nonpeptidergic axons, respectively (Figures 5.2A-5.2M). Finally, 18.9 ± 2.4% of NT5E^{+} neurons express the capsaicin and noxious heat receptor TRPV1 (Figures 5.1P-5.1S and Table 5.1). Together, these imaging studies show that NT5E

protein is in fact expressed in DRG and spinal cord, predominantly in nonpeptidergic, but also in peptidergic nociceptive neurons.

To test if it NT5E degrades AMP in nociceptive circuits, we stained spinal cord as well as DRG sections and cultures from WT and *Nt5e*$^{-/-}$ mice using AMP histochemistry at pH 7.0. In spinal cord, AMP histochemical staining was dramatically reduced in *Nt5e*$^{-/-}$ mice relative to WT in laminae I and II of the dorsal horn, where NT5E^{+} axons terminate (Figure 5.3A-5.3D). This loss of staining in *Nt5e*$^{-/-}$ mice was not due to loss of axon terminals in the dorsal horn (Figure 5.4). In WT DRG sections, staining was most intense in small-diameter neurons, fibers, and the epineural sheath, with some weak granular cytoplasmic staining in large-diameter neurons (Figure 5.3E). In DRG sections from *Nt5e*$^{-/-}$ mice, staining was much less intense in small-diameter and large-diameter neurons, while fiber and epineural staining was almost entirely eliminated (Figure 5.3F). The reduction in staining in *Nt5e*$^{-/-}$ mice was not due to developmental loss of DRG neurons, as wild-type and *Nt5e*$^{-/-}$ mice had equivalent numbers of $P2X_3$-expressing neurons and CGRP-expressing neurons relative to all NeuN^{+} neurons in lumbar ganglia (Table 5.2). In DRG neurons cultured from WT animals, staining is seen predominantly in small-diameter neurons in both the cell body and the projecting neurites (Figure 5.3G). The cellular staining is reduced in cultured DRG neurons from *Nt5e*$^{-/-}$ mice, and strikingly, the neurite staining is completely eliminated (Figure 5.3H). Residual staining in both the DRG and spinal cord is most likely due to PAP activity, which we have previously shown to be an AMPase in these regions (Zylka et al., 2008). Together these data show that NT5E is present in nociceptive neurons and can degrade AMP to locally produce adenosine.

5.3.2) NT5E Protein and AMPase Activity are Reduced in the Spinal Cord Following Nerve Injury

Many genes are up- and down-regulated following nerve injury and can impact the development or maintenance of subsequent neuropathic pain. For example, PAP is downregulated in DRG and spinal cord following nerve injury (Costigan et al., 2002; Davis-Taber and Scott, 2006), and restoring PAP activity can attenuate neuropathic pain behaviors in mice (Zylka et al., 2008). To determine if NT5E protein and activity levels were altered following nerve injury, we conducted immunofluorescence and AMP histochemistry on lumbar spinal cord collected from WT mice fourteen days after unilateral sciatic nerve injury using the spared nerve injury (SNI) model. In this model, the peroneal and sural branches of the sciatic nerve are ligated, while the tibial branch is left intact. This injury results in profound ipsilateral mechanical allodynia and mild thermal hyperalgesia. Strikingly, nerve injury resulted in the complete loss of NT5E protein expression and a decrease in AMPase activity in the regions of lamina II of the spinal cord that receive input from the transected peroneal and sural nerves (Figure 5.5A-5.5E). IB4 labeling was reduced in these regions, while CGRP labeling was unaltered. This loss of NT5E expression and activity following nerve injury could contribute to the subsequent development of mechanical allodynia and thermal hyperalgesia associated with neuropathic pain.

5.3.3) Chronic Pain-induced Thermal Hyperalgesia and Mechanical Allodynia are Enhanced in *Nt5e*$^{-/-}$ Mice.

Since our imaging studies suggest NT5E is expressed in nociceptive neurons, we next tested age-matched WT C57BL/6 and *Nt5e*$^{-/-}$ male mice in a number of acute and chronic

pain behavioral assays. We found no significant differences between genotypes using a measure of acute mechanical sensitivity (electronic von Frey) (Table 5.3). In examining acute thermal sensitivity, we saw no significant differences between the genotypes in the Hargreaves radiant light source or hotplate tests. However, *Nt5e*$^{-/-}$ mice did have shorter latencies in the tail immersion test at both temperatures studied (46.5ºC and 49ºC), indicating an increased acute thermal sensitivity in this assay (Table 5.3). In addition, *Nt5e*$^{-/-}$ mice showed significantly greater thermal hyperalgesia and mechanical allodynia compared to WT in the complete Freund's adjuvant (CFA) model of inflammatory pain (Figures 5.6A, 5.6B). Further, *Nt5e*$^{-/-}$ mice showed significantly greater thermal hyperalgesia in the SNI model of neuropathic pain (Figures 5.6C, 5.6D). Taken together, *Nt5e*$^{-/-}$ mice show only a slight increase in acute thermal sensitivity and no change in acute mechanical sensitivity at baseline, but develop significantly greater thermal hyperalgesia and mechanical allodynia following inflammation or nerve injury. Interestingly, similar patterns of behavior are also seen in *Pap*$^{-/-}$ and A_1-adenosine receptor knockout mice (*$A_1R^{-/-}$*) (Wu et al., 2005; Zylka et al., 2008).

We have recently shown that chronic activation of A_1Rs through the AMPase action of PAP leads to depletion of intracellular stores of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) (Figure 4.8). Conversely, PIP_2 levels are significantly higher in *Pap*$^{-/-}$ mice compared to wild type animals (Figure 4.11A), and this elevation in PIP_2 corresponds with increased responses to pro-nociceptive compounds, including CFA. Since both PAP and NT5E are AMPases that make adenosine and activate A_1Rs, we measured PIP_2 levels in L3-L6 DRG from *Nt5e*$^{-/-}$ and *$A_1R^{-/-}$* mice and compared them to wild type controls. PIP_2 levels were significantly increased in *Nt5e*$^{-/-}$ and *$A_1R^{-/-}$* mice (Figure 5.7), suggesting that loss

of A_1R activation leads to increases in PIP_2, which may be responsible for the enhanced nociception seen in *Nt5e*$^{-/-}$, *A*$_1$*R*$^{-/-}$, and *Pap*$^{-/-}$ mice.

5.3.4) *Nt5e*$^{-/-}$ Mice are Deficient in Degrading AMP in the Spinal Cord *in vivo*

Numerous studies have shown the antinociceptive properties of adenosine and adenosine analogs acting on A_1Rs in the spinal cord (Hayashida et al., 2005; Lavand'homme and Eisenach, 1999; Sawynok, 2007). Further, AMP has antinociceptive properties when infused into the spinal cord in the setting of capsaicin-induced mechanical hyperalgesia (Patterson et al., 2001). This effect was proposed to be due to AMP degradation to adenosine by NT5E and other ectonucleotidases. We thus utilized the antinociceptive effects of AMP to look at the role of NT5E in the degradation of exogenous AMP to adenosine in the spinal cord.

To test this, we measured thermal sensitivity in WT, *Nt5e*$^{-/-}$, and *A*$_1$*R*$^{-/-}$ mice before and after intrathecal (i.t.) injection of a combination of AMP and the adenosine kinase inhibitor 5-iodotubercidin (ITU). Adenosine kinase inhibitors block phosphorylation of adenosine to AMP and prolong the half-life of extracellular adenosine (Kowaluk and Jarvis, 2000). We found it was necessary to combine AMP and ITU, as injection of either compound alone had no effect on thermal sensitivity (Figure 5.9A, 5.9B). Injection of AMP + ITU significantly increased paw withdrawal latency to a noxious thermal stimulus for up to 2 hours in WT and *Nt5e*$^{-/-}$ mice (Figure 5.8A). However, the effect was significantly greater in WT mice compared to *Nt5e*$^{-/-}$ mice, suggesting that NT5E is at least partly responsible for the AMPase activity in WT animals. There was no effect of AMP + ITU in *A*$_1$*R*$^{-/-}$ animals,

showing that adenosine receptor activation is required for the antinociceptive effect of these compounds (Figure 5.8A).

We also examined the effect of AMP + ITU in the setting of ongoing inflammation. We injected one hindpaw of WT and *Nt5e*$^{-/-}$ mice with CFA to induce thermal hyperalgesia. One day later, i.t. injection of AMP + ITU significantly reduced thermal hyperalgesia in the injected paw for up to three hours in WT mice and for up to two hours in *Nt5e*$^{-/-}$ mice (Figure 5.8B). The antinociceptive effect was also significantly less in *Nt5e*$^{-/-}$ mice compared to WT. Taken together, these data show that NT5E is capable of degrading AMP to adenosine to reduce thermal nociception *in vivo*.

5.3.5) Recombinant Mouse NT5E Protein has Long-lasting Antinociceptive Properties through Activation of A_1Rs

We desired to inject a soluble form of NT5E protein to see if it had long-lasting antinociceptive properties similar to soluble PAP (Sowa et al., 2009; Zylka et al., 2008). However, the only source of soluble NT5E protein available was a purified version of the protein from snake venom (Croatalus species, (Aird, 2005)). We were concerned about injecting snake proteins into mice, and thus attempted to make a recombinant version of the mouse NT5E protein using the baculovirus expression system. We have previously used this system to produce large quantities of functional recombinant mouse PAP (Sowa et al., 2009).

We generated a baculovirus expression construct containing a version of NT5E where the C-terminal GPI anchor was removed and replaced by a hexahistidine (H6) epitope tag (mNT5E-His6, henceforth referred to as mNT5E) **(Figure 5.10A, 5.10B).** We detected large quantities of mNT5E protein in the tissue culture supernatant of Hi5 insect cells two days

after infection with recombinant baculovirus. We purified mNT5E from supernatant in one step, using nickel chelate affinity chromatography. We confirmed protein purity by running mNT5E on a SDS-PAGE gel and staining for total protein and western blotting (Figure 5.10C). In both cases, we observed one predominant band at ~60 kDa, corresponding to the calculated molecular weight of mNT5E. This purified, recombinant mNT5E protein effectively dephosphorylated AMP at pH 7.0, indicating the protein is fully functional (Figure 5.10D).

We next tested mNT5E's ability to modulate nociception in vivo. We measured thermal and mechanical sensitivity in WT and $A_1R^{-/-}$ mice before and after initiation of inflammatory pain by intraplantar injection of CFA into one hindpaw. As seen previously (Wu et al., 2005), $A_1R^{-/-}$ mice develop significantly greater thermal hyperalgesia in response to CFA injection (Figure 5.11A). One day later, we injected all animals i.t. with 1.7 units (2.4 μg) mNT5E. In WT mice, mNT5E caused a significant reversal of both thermal hyperalgesia and mechanical allodynia in the injected paw that lasted for 2 days (Figure 5.11A, 5.11B). In addition, paw withdrawal latency also increased in the contralateral, uninjected control paw for up to 2 days. However, mechanical sensitivity was unchanged in the control paw. Thus, mNT5E can reduce thermal nociception in the naïve and inflammatory states, while only affecting mechanical sensitivity in the inflammatory state. No change in thermal or mechanical sensitivity was seen in the inflamed or control paws of $A_1R^{-/-}$ mice after mNT5E injection, suggesting the protein is affecting nociception through activation of A_1Rs. In all cases, mNT5E did not cause paralysis or sedation. Taken together, recombinant mNT5E inhibits nociception through production of adenosine and activation of A_1Rs.

5.4) Discussion

NT5E has been studied in a number of tissues, including colon, kidney, brain, liver, heart, and lung (Colgan et al., 2006; Yegutkin, 2008). Detection of NT5E in these tissues has been largely dependent on enzyme histochemistry, as studies using antibodies directed specifically against NT5E have shown conflicting results (Braun et al., 1994; Schoen et al., 1988; Zimmermann, 1996). These histochemical studies typically use AMP as a substrate at neutral pH. However, this is not specific for NT5E activity, as other enzymes, including PAP, are also capable of degrading AMP under these conditions (Zylka et al., 2008). In the brain, a recent study confirmed that NT5E is expressed broadly in the cortex, hippocampus, and cerebellum using enzyme histochemistry in *Nt5e*$^{-/-}$ mice (Langer et al., 2008). However, such definitive studies have not been done in the spinal cord or DRG. Thus, while NT5E-like activity had been assumed to be present in these regions, prior to our study there was no direct experimental proof that NT5E was expressed and functioned in nociceptive circuits.

In order to address this, we used a commercially available antibody directed against recombinant mouse NT5E protein to show the expression of NT5E in spinal cord and DRG. These studies show that NT5E is indeed expressed in DRG neurons, primarily in the small-diameter, unmyelinated subset. The majority of NT5E^{+} neurons also express markers of the nonpeptidergic subclass of DRG neurons, while a smaller, but still substantial percentage of NT5E^{+} neurons express the peptidergic marker CGRP (Figure 5.1). Fittingly, NT5E protein is seen predominantly in the spinal cord in laminae I and II of the dorsal horn, where peptidergic and nonpeptidergic neurons terminate. Further, these immunofluorescence studies agree with our own enzyme histochemistry studies, which show abundant AMPase

activity in small diameter DRG neurons and the superficial laminae of the dorsal horn. This AMPase activity is severely reduced in *Nt5e*$^{-/-}$ mice, confirming the ability of NT5E to degrade AMP in situ (Figures 5.3A-D). We are thus the first to show definitively that NT5E protein is expressed in nociceptive circuits and is responsible for at least some of the AMPase activity seen in these regions.

Given that NT5E is expressed in nociceptive neurons and can generate adenosine, it is not surprising that genetic deletion of NT5E leads to changes in pain sensation. Indeed, *Nt5e*$^{-/-}$ mice show increased thermal sensitivity in the tail immersion test at baseline and increased thermal hyperalgesia and mechanical allodynia in inflammatory and neuropathic pain states (Figures 5.6A-D). Importantly, this particular phenotype is nearly identical to that seen in *A$_1$R*$^{-/-}$ mice (Wu et al., 2005) and very similar to that seen in *Pap*$^{-/-}$ mice (Zylka et al., 2008). It is thus likely that both NT5E and PAP are acting through a similar mechanism – production of adenosine from the degradation of AMP and subsequent activation of A_1Rs.

NT5E$^+$ neurons are ideally located to act on A_1Rs, as these receptors are concentrated in lamina II of the spinal cord, specifically in close contact with IB4$^+$ neurons and in small- to medium-diameter DRG neurons (Schulte et al., 2003). Thus, NT5E can act locally to generate extracellular adenosine and modulate A_1Rs on presynaptic terminals and postsynaptic neurons in the dorsal horn of the spinal cord. A_1R activation at these sites inhibits presynaptic glutamate release from unmyelinated terminals and inhibits postsynaptic neurons in the substantia gelatinosa of the spinal cord (Lao et al., 2001; Li and Perl, 1994; Patel et al., 2001). In addition, we have recently shown that PAP, through chronic activation of A_1Rs, can deplete intracellular stores of PIP_2 in DRG (Figures 4.8, 4.11). This PIP_2 depletion decreases pro-nociceptive signaling through the noxious heat sensor TRPV1 and

GPCRs. *Pap*$^{-/-}$ mice have increased levels of PIP_2 in their DRG, corresponding with increased nociception. *Nt5e*$^{-/-}$ and *A*$_1$*R*$^{-/-}$ mice also show elevated levels of PIP_2 in DRG compared to wild type animals, again corresponding with increased nociception (Figure 5.7). Whether loss of NT5E activity at A_1Rs is directly leading to this increase in PIP_2 requires further study, but remains a potential mechanism for the inhibition of nociception by endogenous NT5E. Regardless of its exact mechanism of action downstream of A_1Rs, our data suggest that modulation of NT5E activity is a promising new therapeutic approach for the treatment of chronic pain.

5.4.1) The Role of Multiple AMPases in Nociceptive Circuits

Recently, we were the first to identify PAP as an ectonucleotidase in nociceptive circuits (Zylka et al., 2008). PAP is expressed in small-diameter, predominantly nonpeptidergic DRG neurons, degrades AMP to adenosine in vitro and in vivo, and inhibits thermal hyperalgesia and mechanical allodynia induced by inflammation or nerve injury through activation of A_1Rs. In the course of our studies, we recognized that while PAP was partly responsible for the visible AMPase activity in DRG and lamina II of spinal cord, some of this activity remained in *Pap*$^{-/-}$ mice, suggesting there is at least one additional ectonucleotidase in these regions. Here, we have shown that NT5E is largely responsible for this remaining AMPase activity. Somewhat surprisingly, not only is NT5E expressed in these regions, but its expression pattern closely mimics that of PAP. Like PAP, NT5E is found predominantly in nonpeptidergic DRG neurons, as defined by the markers $P2X_3$, IB4, and *MrgprD*. In fact, virtually all (95.5 ± 1.1%) NT5E$^+$ neurons also express PAP, while the vast majority of PAP$^+$ neurons also express NT5E (82.7 ± 1.5%). In addition, NT5E and

PAP expression overlap significantly in the dorsal horn of the spinal cord, although PAP seems to be limited to lamina II, while NT5E extends slightly into lamina I as well.

Why is there overlap in the expression of two enzymes that seemingly perform the same action, namely degradation of AMP to adenosine? While both NT5E and PAP can indeed dephosphorylate AMP, their functional profiles are very different. NT5E hydrolyses exclusively 5'-monophosphates, with 5'-AMP being its favored substrate (Zimmermann, 1992). Its K_m for AMP is in the low micromolar range and its pH optimum is somewhere between 7 and 8 (Zimmermann, 1992). PAP, on the other hand, is capable of dephosphorylating numerous substrates, including para-nitrophenyl phosphate (p-NPP), β-glycerophosphate, lysophosphatidic acid, and many nucleotides, especially nucleotide monophosphates (AMP, TMP, XMP, CMP, GMP, IMP, and UMP) (Ostrowski and Kuciel, 1994; Silverman and Kruger, 1988a). Among adenine-containing nucleotides, PAP can dephosphorylate AMP, ADP, and ATP at acidic pH (with relative activity AMP > ADP > ATP), but predominantly acts on AMP at neutral pH (Sowa et al., 2009). The K_m of PAP for AMP at neutral pH is in the low millimolar range, and the enzyme has activity over a very broad pH range (pH 4 – 8) (Dziembor-Gryszkiewicz et al., 1978; Lam et al., 1973; Sowa et al., 2009).

Thus, while both NT5E and PAP are capable of degrading AMP, their functional properties hint at different roles depending on the conditions outside of the cell. At normal extracellular pH, it seems NT5E is much better suited to rapidly degrade AMP to adenosine, as it has a much greater affinity for AMP than PAP under these conditions. However, when external pH deviates from neutral, PAP is still capable of making adenosine, while NT5E is not. This is particularly important considering that tissue injury produces an "inflammatory

soup" containing protons and nucleotides that can reduce extracellular pH (Julius and Basbaum, 2001). Under "resting" conditions NT5E may be more important than PAP in the degradation of AMP, but PAP might play a more vital role following repeated neuronal activation or inflammation. Studies of AMP degradation under different conditions *in vivo* in mice lacking one or both of the enzymes might help clarify their specific roles.

In addition, while NT5E is limited in its activity to 5'-nucleoside monophosphates, PAP is much more promiscuous and might also be important in the dephosphorylation of other substrates. For example, PAP is capable of directly degrading pro-nociceptive compounds like lysophosphatidic acid and creating antinociceptive compounds like 2-arachidonylglycerol (Hillard, 2000; Nakane et al., 2002; Sugiura et al., 2000; Tanaka et al., 2004). While our previous studies pointed to PAP's AMPase activity as the main source of its antinociceptive action, it is possible PAP is also important in the degradation of pro-nociceptive compounds or the production of antinociceptive compounds under conditions we haven't examined.

Finally, while our data suggest that NT5E and PAP are the main AMPases present in the DRG and dorsal horn of the spinal cord, the possibility remains that there are other ectonucleotidases in these regions that could also make adenosine from AMP. For example, alkaline phosphatases are non-specific phosphomonoesterases which are capable of degrading nucleoside 5'-tri-, -di-, and –monophosphates, including AMP (Zimmermann, 2006). Tissue-nonspecific alkaline phosphatase (TNAP) is expressed in the spinal cord, but its detailed expression and role in modulation of nociceptive signaling has not been studied. Further, while AMP conversion to adenosine is the rate-limiting step in the pathway of degradation of ATP to adenosine, other ectonucleotidases, such as E-NTPDases, are capable

of catalyzing the upstream reactions of ATP and/or ADP dephosphorylation (Zimmermann, 2006). These enzymes have not been studied in the nociceptive system, and given the importance of adenine nucleotides in pain signaling, they could also be important targets for the treatment of chronic pain.

5.4.2) Therapeutic Potential of Recombinant NT5E

A promising and exciting new approach for the treatment of chronic pain is the use of recombinant NT5E protein. Here we have shown that purified recombinant mouse NT5E is has long-lasting antinociceptive properties. In fact, a single i.t. injection lasted for several days and was antihyperalgesic specifically in the thermal modality in uninjured animals and was antihyperalgesic and antiallodynic in CFA-inflamed animals. These effects were dependent on A_1R activation, confirming that recombinant mNT5E acts through the production of adenosine from AMP. The nature and duration of the antinociceptive effects of mNT5E are similar to purified human PAP (hPAP) and recombinant mouse PAP (mPAP), both of which also act through activation of A_1Rs (Sowa et al., 2009; Zylka et al., 2008). Amazingly, like hPAP and mPAP, a single injection mNT5E has antinociceptive properties that are similar in magnitude but much longer in duration than the commonly used analgesic morphine (Zylka et al., 2008).

Somewhat surprising is the absence of obvious side effects following injection of mNT5E. While adenosine A_1R agonists have antinociceptive properties in humans and in animal models of chronic inflammatory and neuropathic pain (Hayashida et al., 2005; Lavand'homme and Eisenach, 1999; Sawynok, 2007), they are not clinically used due to severe side effects, including transient lower back pain and motor paralysis at high doses

(Belfrage et al., 1999; Eisenach et al., 2003; Sawynok, 2007). Motor side effects are not surprising, given the high expression of A_1Rs in motor neurons (Reppert et al., 1991). While mNT5E presumably produces adenosine and activates A_1Rs throughout the spinal cord, we do not see motor effects at the dose of mNT5E tested. This could be due to the fact that, as an enzyme, the amount of adenosine produced by mNT5E is limited by the substrate concentration, in this case AMP. Thus, mNT5E may be producing a sufficient amount of adenosine in the dorsal horn of the spinal cord to mediate antinociception, but not enough to inhibit motor function in the ventral horn. This catalytic restriction could also explain the absence of motor side effects following injection of hPAP or mPAP (Sowa et al., 2009; Zylka et al., 2008).

Recombinant proteins are routinely used to treat a number of human diseases and disorders. These proteins are relatively easy to produce and purify and are safe to use in humans. Thus, it is likely that a recombinant human form of NT5E could be produced as a treatment for not only chronic pain, but also for a number of other conditions where production of adenosine is important in mediation of the disease process. In fact, administration of NT5E is beneficial in other experimental scenarios. For example, soluble NT5E promotes vascular barrier function and decreases neutrophil accumulation in inflammatory models of lung disease, reduces macrophage trafficking in ischemic brain tissue, protects renal tissue from ischemic injury, and decreases myocardial infarct size through ischemic preconditioning (Eckle et al., 2007b; Eltzschig et al., 2004; Grenz et al., 2007; Petrovic-Djergovic et al., 2007; Reutershan et al., 2009; Thompson et al., 2004b). The NT5E used in these studies is not human, but rather collected from the venom of rattlesnakes (typically from the genera *Crotalus*) (Aird, 2005). Obviously, the development of a

recombinant form of the human NT5E protein would be much more desirable for therapeutic purposes in humans. Such a protein, when delivered locally, could play an important role in the treatment not only of chronic pain, but of many disease processes, including myocardial ischemia/infarction, stroke, acute lung injury, and renal failure.

5.5) Figures and Tables

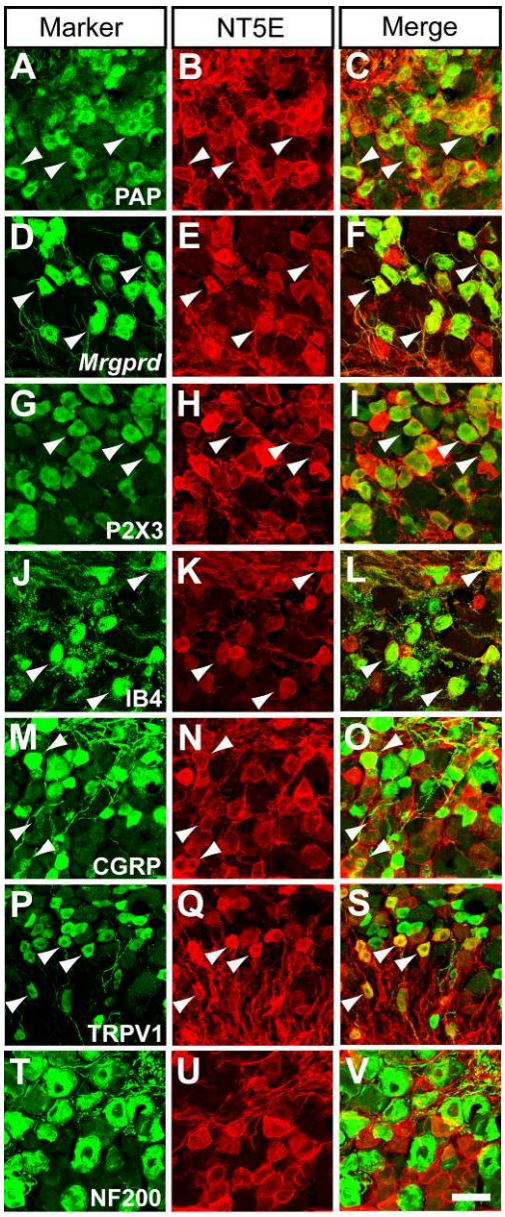

Figure 5.1. NT5E is expressed primarily in nonpeptidergic DRG neurons. (A-V) Mouse L4-L6 DRG neurons were stained with antibodies against various sensory neuron markers (green) and with antibodies against NT5E (red). Arrowheads mark examples of double-labeled cells. Images were acquired by confocal microscopy. Scale bar (see panel V) is 50 μm for all panels. (Back to text)

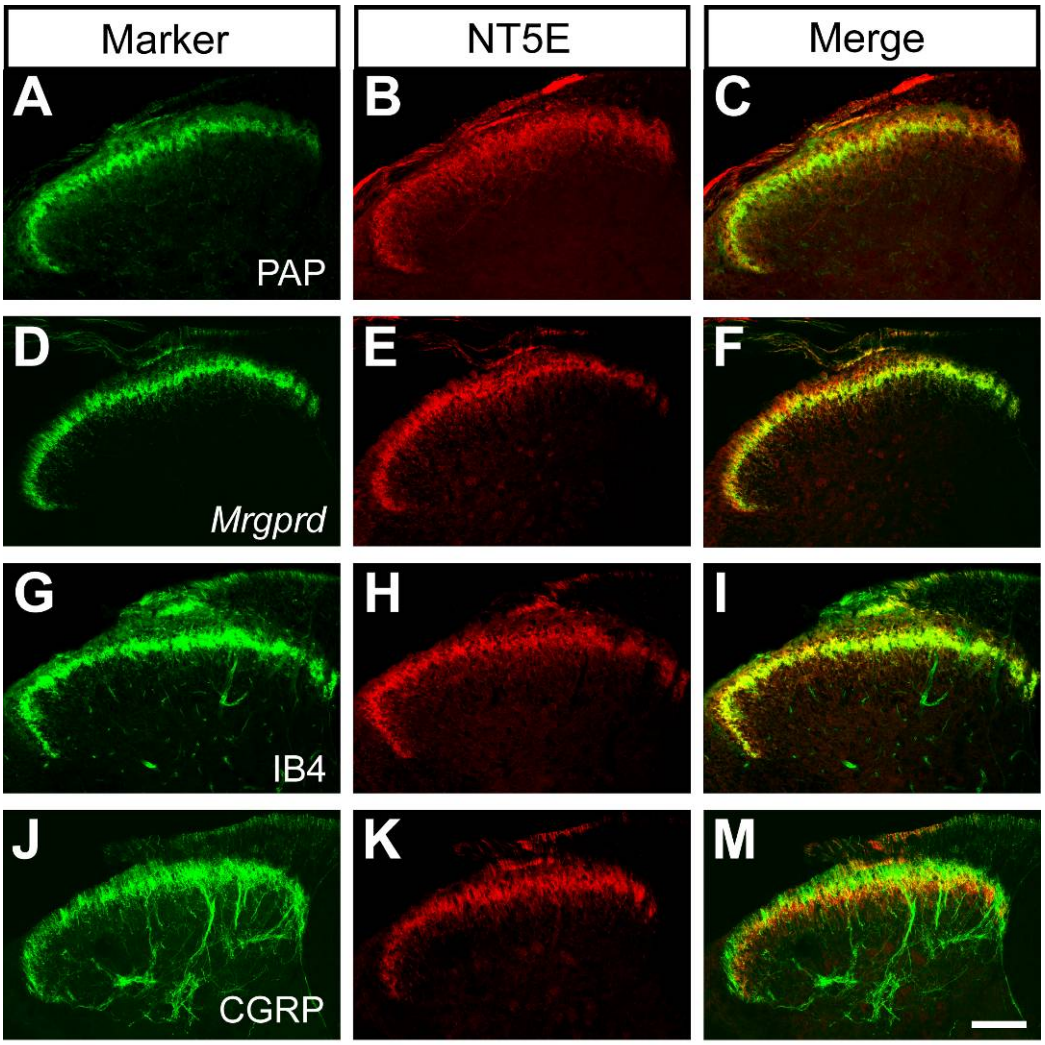

Figure 5.2. NT5E protein is localized primarily to nonpeptidergic and some peptidergic axon terminals in dorsal spinal cord. Lumbar spinal cord sections were double-labeled with antibodies against selected axonal markers (A, D, G, J; green) and NT5E (B, E, H, K; red). IB4, *Mrgprd*Δ_{EGFPf}, and PAP mark nonpeptidergic endings in lamina II. CGRP marks peptidergic endings in lamina I. Images were acquired by confocal microscopy. Scale bar: 150 μm for all panels. (Back to text)

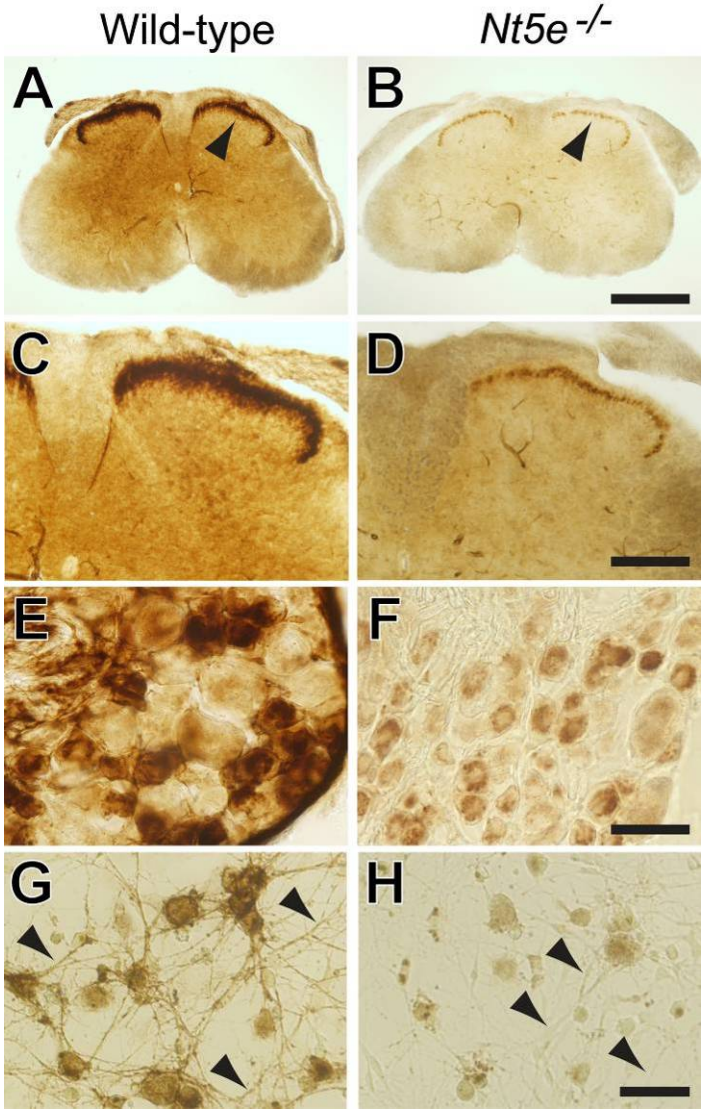

Figure 5.3. NT5E has AMPase activity in nociceptive circuits. (A-D) Spinal cord, (E, F) lumbar DRG, and (G, H) cultured DRG neurons from wild-type (WT) and *Nt5e*$^{-/-}$ adult mice stained with AMP histochemistry. (A, B) Staining was primarily localized to the dorsal horn (arrowhead) of spinal cord, and was diminished in *Nt5e*$^{-/-}$ mice. (C, D) are the same spinal cords from (A, B) at higher magnification. (E, F) Staining was diminished in cell bodies of DRG neurons and eliminated in fibers and epineurium in *Nt5e*$^{-/-}$ mice. (G, H) Staining was diminished in DRG cell bodies and eliminated in neurites in *Nt5e*$^{-/-}$ mice (arrowheads). AMP

(3 mM in [A-D] and 6 mM in [E-H]) was used as substrate, and buffer was pH 7.0. Scale bar is 500 um in (A) and (B), 200 um in (C) and (D), and 50 um in (E-H). (Back to text)

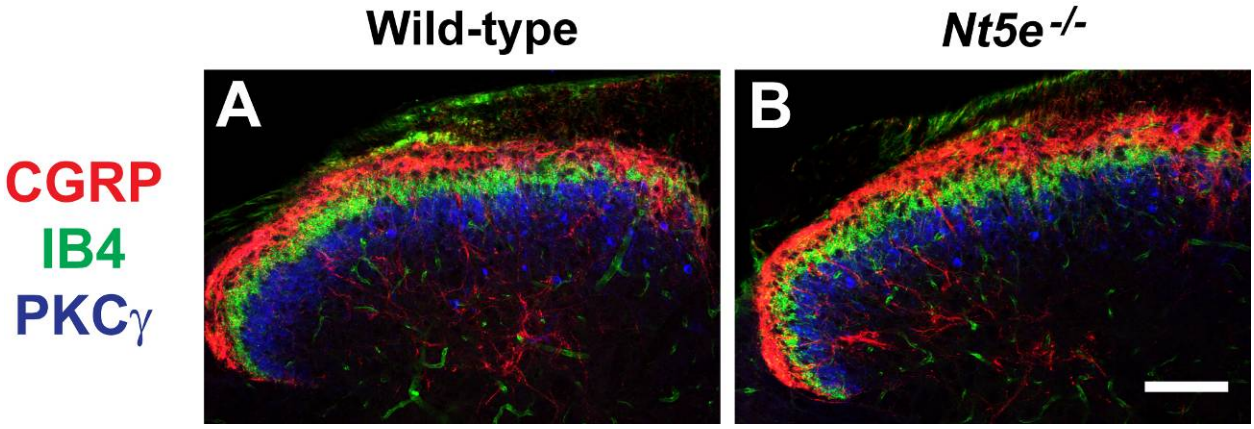

Figure 5.4. Axon terminals are anatomically normal in *Nt5e*$^{-/-}$ mice. Lumbar spinal cord sections from (A) wild-type and (B) *Nt5e*$^{-/-}$ mice were stained with antibodies to CGRP (to mark peptidergic nerve endings), isolectin B4 (IB4, to mark nonpeptidergic nerve endings) and antibodies to protein kinase C-γ (PKCγ, to mark interneurons in laminas II$_{inner}$ and III). Confocal image analysis revealed no gross anatomical differences between genotypes (n=2 mice from each genotype). Scale bar: 100 μm. (Back to text)

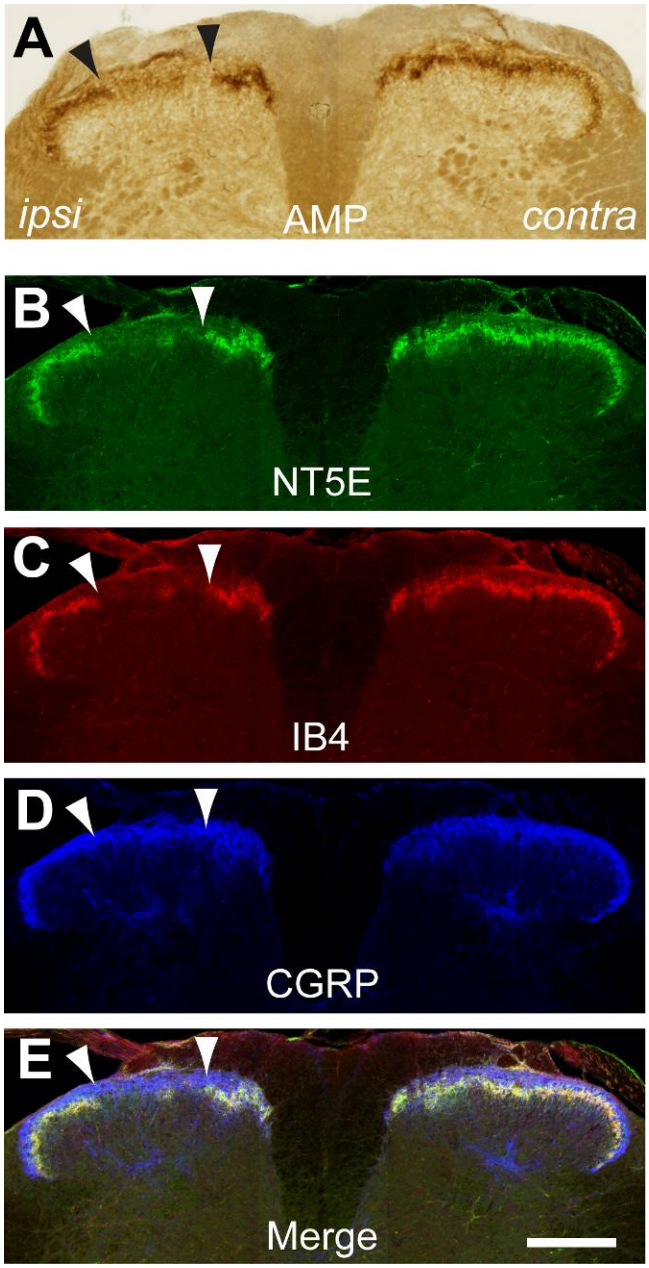

Figure 5.5. NT5E protein and activity is reduced following peripheral nerve injury. (A-E) Lumbar spinal cord from a wild-type mouse (A) stained using AMP histochemistry or labeled with antibodies against the indicated axonal markers 14 days after unilateral injury to the left peroneal and sural nerves. The region of the spinal cord receiving innervation from these injured nerves (arrowheads) showed reduced staining and labeling. For (A) AMP (3 mM) was used as substrate and buffer pH was 7.0. Scale bar 200 μM. (Back to text)

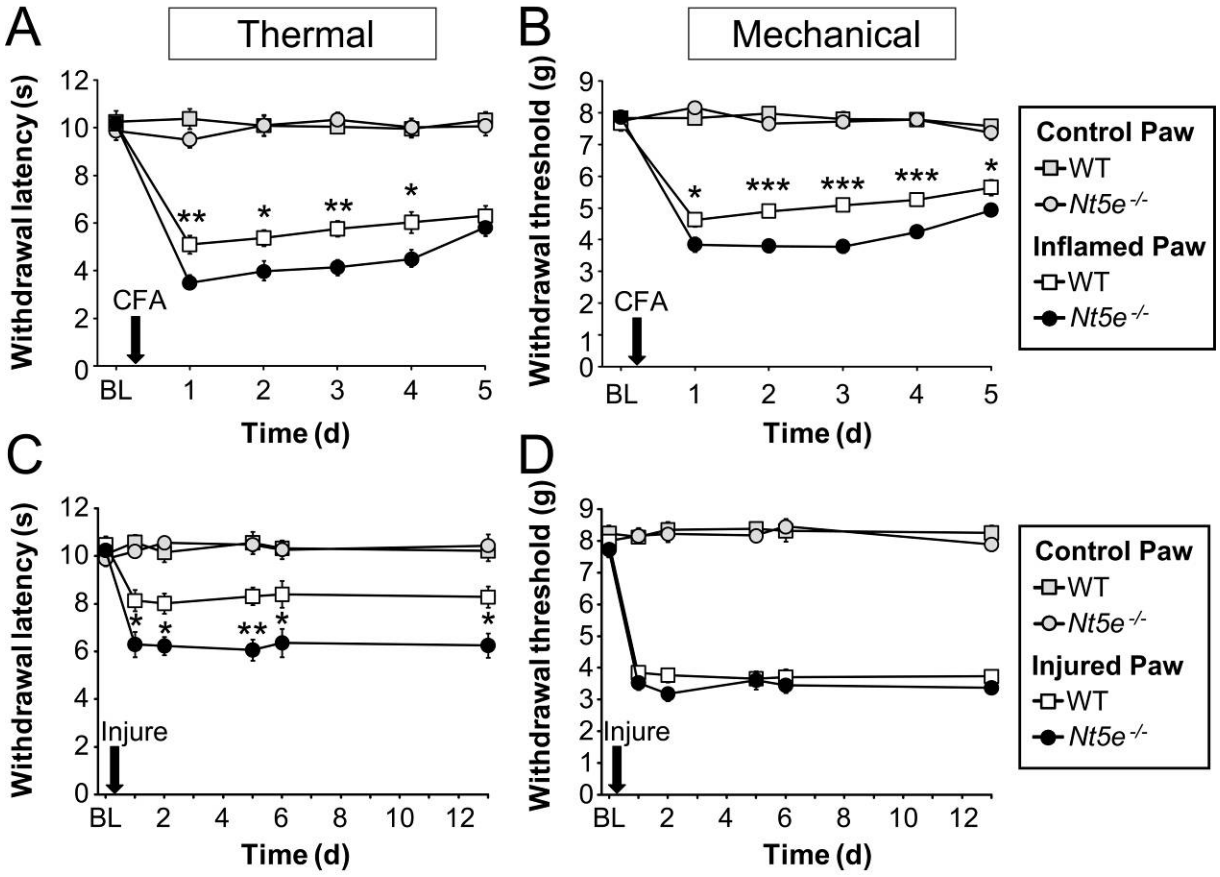

Figure 5.6. ***Nt5e*$^{-/-}$ mice show enhanced nociceptive responses following inflammation and nerve injury.** (A, B) CFA inflammatory pain model. Wild-type and *Nt5e*$^{-/-}$ mice were tested for (A) thermal and (B) mechanical sensitivity before (BL) and following injection of CFA (arrow) into one hindpaw. The contralateral hindpaw served as control. (C, D) SNI neuropathic pain model. Wild-type and *Nt5e*$^{-/-}$ were tested for (C) thermal and (D) mechanical sensitivity prior to (BL) and after ligation and transaction of the sural and common peroneal branches of the sciatic nerve. (A-D) Paired t tests were used to compare responses at each time point between wild-type and *Nt5e*$^{-/-}$ mice (n = 10 per genotype); same paw comparisons. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005. All data are presented as means ± s.e.m. (Back to text)

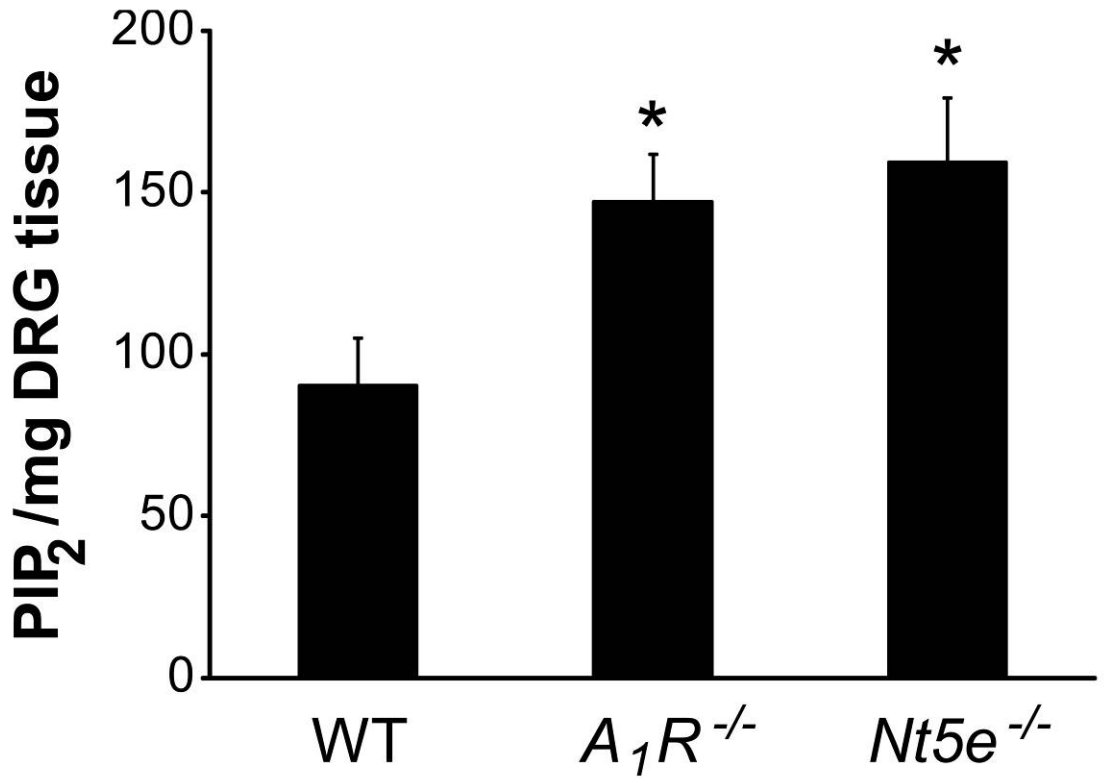

Figure 5.7. PIP_2 levels are increased in $A_1R^{-/-}$ and $Nt5e^{-/-}$ mice. PIP_2 levels in L3-L6 DRG were quantified in wild-type (WT) mice, $A_1R^{-/-}$ mice, and $Nt5e^{-/-}$ mice. n=3 mice per condition. $^*P < 0.05$ compared to WT by paired t test. Values are means ± s.e.m. (Back to text)

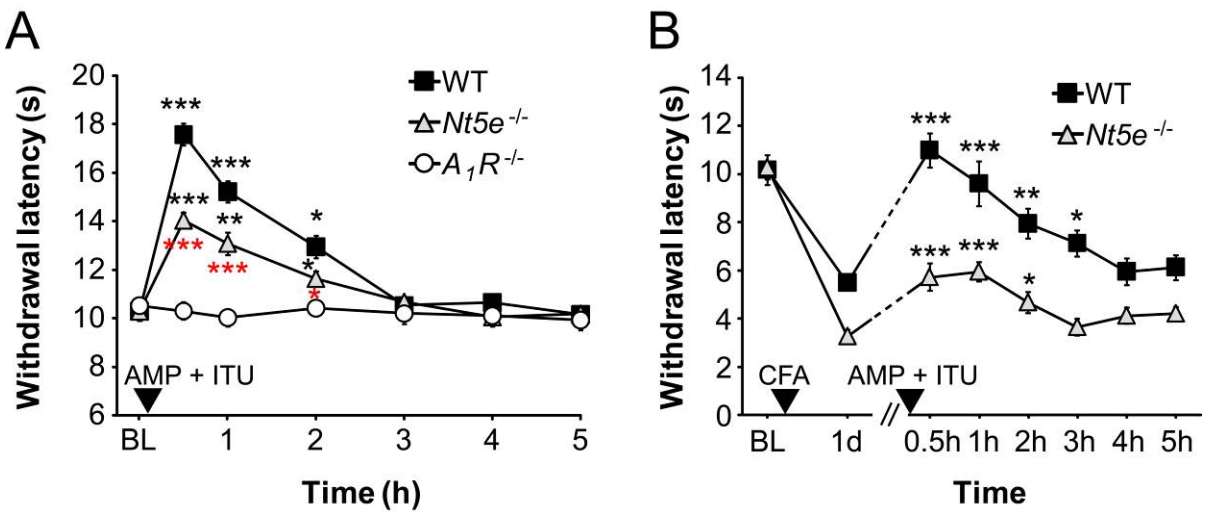

Figure 5.8. NT5E degrades AMP to adenosine in vivo. (A) AMPase-induced antinociception in wild-type (WT), *Nt5e*$^{-/-}$, and *A1R*$^{-/-}$ mice. Thermal sensitivity was measured at baseline (BL) and after i.t. injection of AMP (200 nmol) + 5-iodotubercidin (ITU; 5 nmol). Paired t tests were used to compare responses at each timepoint to BL values (black asterisks) within a given genotype (n = 8 mice per genotype). Paired t tests were also used to compare responses at each time point between WT and *Nt5e*$^{-/-}$ mice (red asterisks). (B) AMPase-induced antinociception in WT and *Nt5e*$^{-/-}$ mice following inflammation. Mice were tested for thermal sensitivity before (BL) and after injection of CFA (arrowhead) into one hindpaw. One day later, all mice were injected i.t. with AMP (200 nmol) + ITU (5 nmol) and thermal sensitivity was measured for several hours after injection. Paired t tests were used to compare responses following AMP + ITU injection to the response 1 day after CFA injection in the same paw. n = 8 mice per genotype. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005. All data are presented as means ± s.e.m. (Back to text)

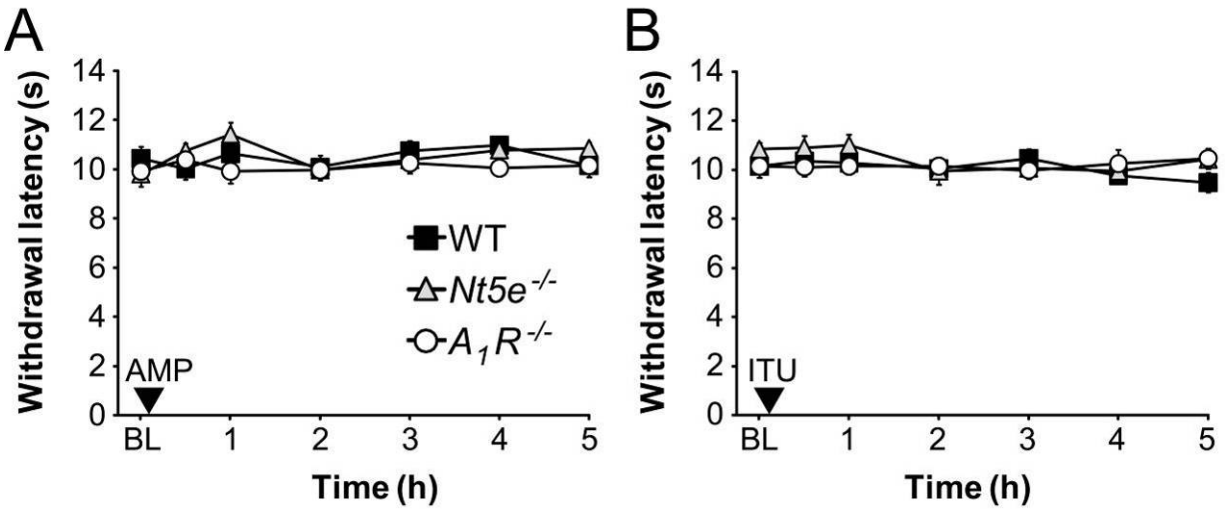

Figure 5.9. Injection of AMP or 5-iodotubercidin (ITU) alone has no effect on thermal sensitivity. Thermal sensitivity was measured before (baseline, BL) or after intrathecal (i.t.) injection with (A) AMP (200 nmol) or (B) ITU (5 nmol). No significant differences from BL values were seen in any genotype. n = 8 mice per genotype. All data are presented as means ± s.e.m. (Back to text)

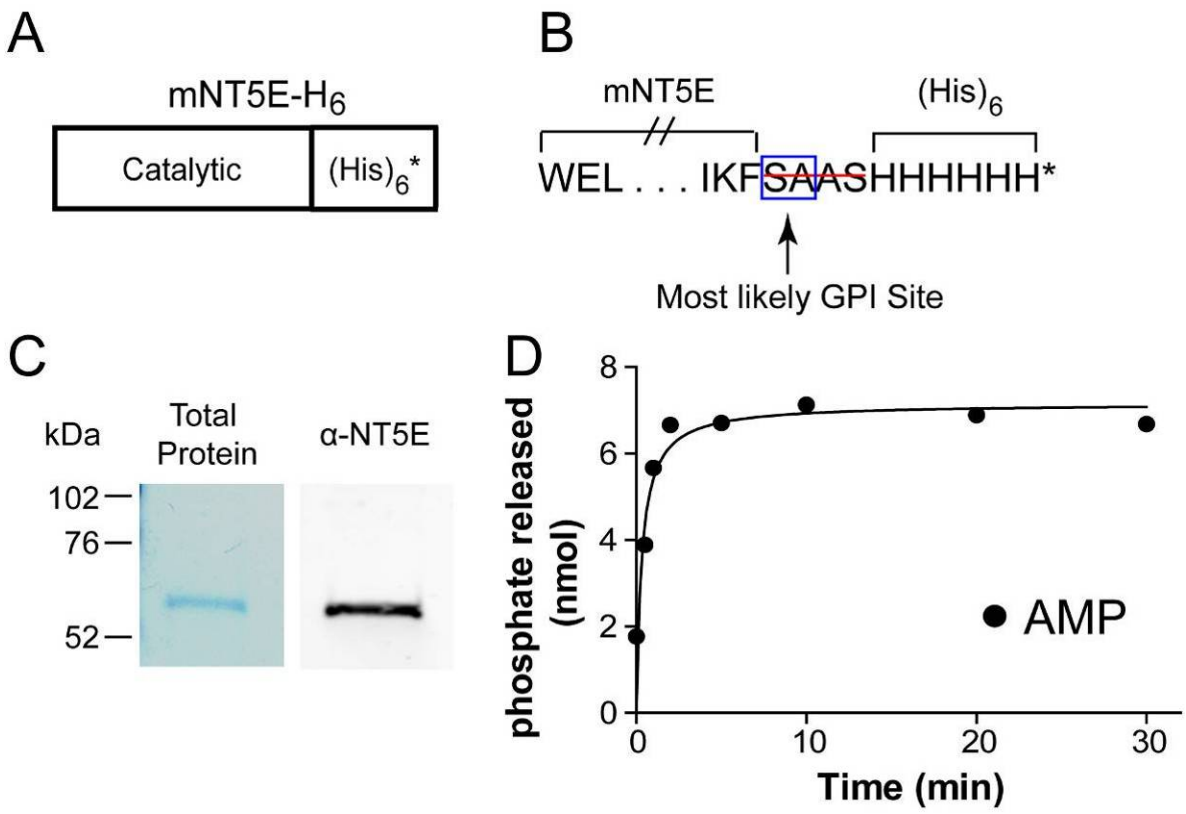

Figure 5.10. Purification of recombinant mNT5E. (A) A hexahistidine tag (H6) and stop codon (*) were added to the C-terminus of mouse NT5E. Map is not drawn to scale. (B) Amino acid sequence at the junction between the catalytic domain and H6 tag. A four amino-acid putative GPI anchor site (arrow) was removed at the C-terminus, just upstream of the H6 tag. Asterisk marks stop codon. (C) GelCode blue-stained SDS-PAGE gel and western blot of purified recombinant mNT5E protein (1 mg and 5 mg, respectively). The western blot was probed with an anti-NT5E antibody. (D) AMP (400 μM) degradation by mNT5E as measured by moles of released inorganic phosphate over time. (Back to text)

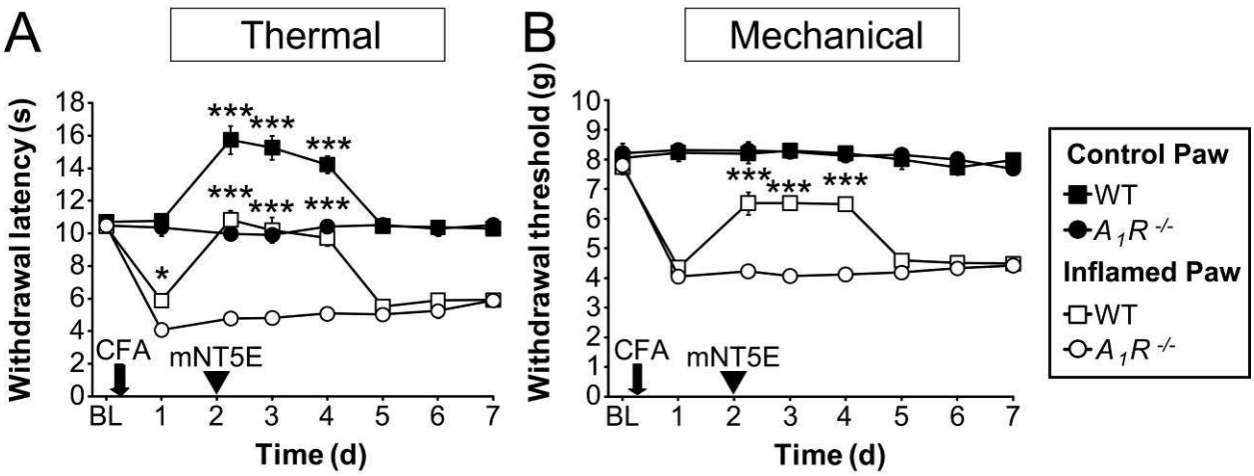

Figure 5.11. mNT5E protein has long-lasting antinociceptive effects when injected intraspinally. Wild-type (WT) and $A_1R^{-/-}$ mice were tested for (A) thermal and (B) mechanical sensitivity before (baseline, BL) and after injection of CFA into one hindpaw (arrow). One day later, mNT5E protein (1.7 units) was injected i.t. into all mice (arrowhead) and thermal sensitivity was measured for several days. Inflamed and non-inflamed (control) hindpaws were both measured. Paired t testes were used to compare responses at each time point between genotypes (n = 10 animals per genotype). $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

Table 5.1. Quantitative analysis of NT5E and sensory neuron marker co-localization within wild-type mouse L4-L6 DRG neurons.

Marker	Percentage of $NT5E^+$ neurons expressing indicated marker	Percentage of $marker^+$ neurons expressing NT5E
PAP	**95.5 ± 1.1**	**82.7 ± 1.5**
$P2X_3$	**89.3 ± 1.0**	**82.3 ± 1.9**
IB4	**83.6 ± 2.6**	**87.4 ± 2.5**
MrgprD-EGFPf	**74.9 ± 1.4**	**98.5 ± 0.3**
CGRP	**44.6 ± 3.6**	**38.0 ± 2.4**
TRPV1	**18.9 ± 2.4**	**26.1 ± 3.0**
NF200	**4.5 ± 0.9**	**4.4 ± 0.9**

At least 500 cells were counted per combination. Data are means ± s.e.m.
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Table 5.2. Comparison of CGRP-containing and $P2X_3$-containing populations in L4-L6 DRG from wild-type and *Nt5e*$^{-/-}$ mice.

	% $CGRP^+$	% $P2X_3^+$
Wild-type	**45.0 ± 1.6**	**45.3 ± 1.2**
Nt5e$^{-/-}$	**44.5 ± 0.9**	**44.6 ± 1.1**
	P = 0.782	*P = 0.685*

Percentage of total $NeuN^+$ neurons expressing the markers was calculated. Dorsal root ganglia from three mice counted per genotype. Counter was blind to genotype. Data expressed as means ± s.e.m.
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Table 5.3. Acute mechanical and thermal sensitivity in *Nt5e*$^{-/-}$ mice.

Behavioral Assay	Wild-type	*Nt5e*$^{-/-}$
	Withdrawal threshold:	
Electronic von Frey	8.1 ± 0.2 g	7.7 ± 0.2 g
	Withdrawal latency:	
Radiant heating of hindpaw (Hargreaves Method)	10.6 ± 0.3 s	10.3 ± 04 s
Tail immersion at 46.5°C	13.8 ± 1.1 s	9.5 ± 0.5 s**
Tail immersion at 49.0°C	5.6 ± 0.3 s	4.2 ± 0.2 s**
Hot plate at 52°C	29.4 ± 2.1 s	31.4 ± 2.7 s
Hot plate at 55ºC	15.3 ± 1.9 s	15.8 ± 1.5 s

Data are expressed as means ± s.e.m. Paired t-tests were used to compare genotypes for each test, $^{**}P < 0.005$. n=10 male mice tested per genotype.
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CHAPTER 6

Conclusions and Discussion

6.1) Summary of Findings

The major findings presented in this work include:

Prostatic Acid Phosphatase Suppresses Pain by Generating Adenosine

1) Prostatic acid phosphatase (PAP) is the molecular identity of the fluoride resistant acid phosphatase (FRAP)/TMPase present in dorsal root ganglia and spinal cord.
2) PAP is expressed predominantly in the nonpeptidergic class of nociceptive DRG neurons whose axons terminate in lamina II of the dorsal spinal cord.
3) PAP colocalizes extensively with IB4, $P2X_3$, and *MrgprD* and only minimally with CGRP in DRG. There is some overlap with TRPV1.
4) Genetic deletion of PAP does not affect acute nociception.
5) Genetic deletion of PAP leads to greater thermal hyperalgesia and mechanical allodynia following inflammatory insult.
6) Genetic deletion of PAP leads to greater thermal hyperalgesia following nerve injury.
7) Intrathecal injection of soluble PAP leads to dose-dependent thermal antinociception in naïve mice that lasts for up to three days.
8) Intrathecal injection of soluble PAP partially reverses thermal hyperalgesia and mechanical allodynia in inflammatory and neuropathic pain models.

9) PAP acts as an AMP-degrading ectonucleotidase in vitro, in situ, and in vivo.
10) PAP's antinociceptive effect depends on activity at A_1-adenosine receptors.
11) PAP is the first extensively characterized ectonucleotidase in nociceptive circuits.

Recombinant Mouse PAP has pH-Dependent Ectonucleotidase Activity and Acts through A_1-Adenosine Receptors to Mediate Antinociception

1) Large quantities of recombinant mouse PAP (mPAP) can be made using the baculovirus expression system.
2) mPAP is an ectonucleotidase in vitro, capable of degrading AMP > ADP > ATP at pH 5.6 and AMP at pH 7.0.
3) Intrathecal injection of mPAP has dose-dependent thermal antinociceptive effects in naïve mice that last for three days.
4) Intrathecal injection of mPAP partially reverses thermal hyperalgesia and mechanical allodynia in inflammatory pain.
5) mPAP's antinociceptive action depends on activity of A_1R, suggesting it acts as an ectonucleotidase in vivo.

Prostatic Acid Phosphatase Reduces Pain Sensitization and TRPV1-dependent Thermal Sensitivity by Depleting PIP_2

1) PAP inhibits activity of the noxious heat and capsaicin receptor TRPV1 in vitro and in vivo.
2) PAP's action on TRPV1 depends on its ability to act as an ectonucleotidase and activate A_1R.

3) PAP's thermal antinociceptive effects when injected intrathecally are partially due to inhibition of TRPV1.
4) Genetic deletion of PAP enhances capsaicin-evoked pain responses.
5) PAP activation of A_1R leads to depletion of cellular levels of PIP_2 in vitro through a PLC-dependent process.
6) PAP-mediated depletion of cellular PIP_2 reduces TRPV1 activity in vitro.
7) Genetic deletion of PAP increases levels of PIP_2 in DRG in vivo, while injection of PAP decreases levels of PIP_2 in DRG.
8) PAP's antinociceptive effects in vivo are blocked by inhibition of PLC or direct addition of PIP_2.
9) PAP inhibits signaling through pro-nociceptive GPCRs by activation of A_1R and depletion of cellular PIP_2 in vitro.
10) PAP blocks ATP-, LPA-, and nerve-injury induced hyperalgesia and allodynia.

NT5E is Expressed in Nociceptive Circuits and Suppresses Pain by Generating Adenosine

1) NT5E is expressed predominantly in nonpeptidergic nociceptive neurons and in some peptidergic neurons in DRG that terminate in laminae I and II in the dorsal spinal cord.
2) NT5E is responsible for some of the AMP-degrading ectonucleotidase activity in the DRG and dorsal spinal cord in situ and in vivo.
3) NT5E expression and activity is reduced following peripheral nerve injury.
4) Genetic deletion of NT5E increases PIP_2 levels in DRG.
5) Genetic deletion of NT5E increases acute thermal nociception.

6) Genetic deletion of NT5E increases thermal hyperalgesia and mechanical allodynia following inflammation and increases thermal hyperalgesia following nerve injury.
7) NT5E degrades AMP to adenosine and suppress nociception in vivo.
8) Large quantities of functionally active recombinant mouse NT5E (mNT5E) can be made using the baculovirus expression system.
9) Intrathecal injection of mNT5E leads to thermal antinociceptive effects in naïve mice that last for two days.
10) Intrathecal injection of mNT5E partially reverses thermal hyperalgesia and mechanical allodynia following inflammation.
11) mNT5E's antinociceptive effects depend on the presence of A_1R, suggesting it acts as an AMPase in vivo.

6.2) Multiple AMPases in Nociceptive Circuits

The work presented here represents the first known attempt to molecularly identify and characterize ectonucleotidases present in nociceptive circuits. Given the important role of adenine-containing nucleotides in the modulation of nociception, it is surprising no one has previously systematically studied these enzymes. While ectonucleotidase activity had been shown to be present in nociceptive circuits through several different lines of evidence, no follow-up had occurred in the cloning era to more precisely identify these important genes. Our work not only molecularly identifies the first two known ectonucleotidases in nociceptive circuits (see Figure 1.5, red text), but also demonstrates the importance of these enzymes in the modulation of nociceptive signaling. Further, we demonstrate the potential for targeting these enzymes for novel therapeutic approaches.

Both PAP and NT5E are expressed in similar neuronal populations in the DRG, and are present in axon terminals with overlapping expression in the dorsal horn. In fact, virtually all (95.5 ± 1.1%) $NT5E^+$ neurons also express PAP, while the vast majority of PAP^+ neurons (82.7 ± 1.5%) also express NT5E. This high degree of overlap is somewhat surprising given both enzymes inhibit nociception through the same mechanism: degradation of AMP to adenosine and subsequent activation of A_1R. This apparent shared pathway is demonstrated by the similar phenotypes seen in $PAP^{-/-}$, $Nt5e^{-/-}$, and $A_1R^{-/-}$ mice (Figure 2.8; Wu et al., 2005). All three knockout strains show minimal thermal nociceptive deficits in naïve animals, and significantly greater thermal hyperalgesia following inflammation and nerve injury.

It is of special note that deletion of a single ectonucleotidase enzyme produces a phenotype in these models that is identical to that seen in $A_1R^{-/-}$ mice. This suggests that loss of one enzyme is enough to reduce DRG and spinal adenosine production below that necessary to activate anti-nociceptive A_1R in the setting of inflammation or nerve injury. This result is somewhat surprising and could demonstrate a real physiologic effect or could reflect an inability of our measures to tease out small differences in thermal or mechanical sensitivity. Indeed, the tools we use for mechanical (electronic Von Frey) and thermal (radiant light source; Hargreaves) sensitivity testing are unlikely to pick up very subtle differences between animals. Regardless, our results demonstrate that endogenous PAP and NT5E act through similar pathways to reduce nociception.

This similarity of action is also demonstrated in the antinociceptive effects seen following intraspinal injection of soluble forms of the PAP and NT5E proteins. Both enzymes produce selective thermal antinociception in naïve animals and can reverse thermal

hyperalgesia and mechanical allodynia following inflammation. Their antinociceptive effects are similar in onset, magnitude, and duration and require activity of A_1R, suggesting a similar mechanism of action (namely degradation of AMP to adenosine). Notably, A_1R agonists also produce antinociceptive effects that are selective for the thermal modality in naïve animals, while decreasing both thermal and mechanical hyperalgesia in inflamed or nerve-injured animals (Dickenson et al., 2000; Sawynok, 1998). There are also reports of long-lasting antinociceptive effects following intraspinal delivery of adenosine or adenosine receptor agonists (Belfrage et al., 1995; Eisenach et al., 2003), similar to what we see with soluble PAP and NT5E.

Unlike adenosine or A_1R agonists, PAP and NT5E do not produce motor side effects when given intrathecally. This is likely due to the fact that as enzymes, PAP and NT5E are restricted in their activity by the abundance of their substrate, namely AMP. It seems likely that both enzymes are producing adenosine throughout the spinal cord, but while the 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 lower levels of endogenous AMP. This catalytic restriction may limit the amount of A_1R activation and subsequent antinociception capable with PAP or NT5E administration, but could be advantageous by avoiding unwanted side-effects.

Perhaps the most revealing findings concerning the functional redundancy of PAP and NT5E come from our experiments examining AMP-induced antinociception in vivo. When injected intrathecally in conjunction with an adenosine kinase inhibitor, AMP produces a transient, thermal-selective antinociceptive effect. This effect is dependent upon activation of A_1R, suggesting it is due to AMP degradation to adenosine in vivo by neuronal

ectonucleotidases. Interestingly, AMP injection still results in antinociception in *Pap*$^{-/-}$ and *Nt5e*$^{-/-}$ mice, although the effect is significantly lower than that seen in WT animals (Figures 5.8A, 6.1). The knockout strains have very similar phenotypes, suggesting the enzymes have similar abilities to degrade AMP in vivo. Amazingly, genetic deletion of both enzymes (*Nt5e*$^{-/-}$; *Pap*$^{-/-}$) leads to almost a complete inability to degrade AMP to adenosine and suppress acute nociception in vivo , suggesting the vast majority of the effect is due to these two enzymes (Figure 6.1). However, a minor antinociceptive effect is seen in *Nt5e*$^{-/-}$; *Pap*$^{-/-}$ mice, suggesting there is still some AMPase activity in the spinal cords of these animals. This effect is relatively minor, and could be due to alkaline phosphatases, which are also expressed in nociceptive circuits (see Section 1.5.4.3). Regardless of this minor effect, our experiments suggest that PAP and NT5E have similar efficacies in degrading AMP to adenosine in vivo, and that these two enzymes are the most important AMPases in nociceptive circuits.

The most obvious question that arises from these results is why does the nociceptive nervous system contain two enzymes in almost the exact same population of neurons that perform the same function. As discussed in Chapter 5, there could be two major reasons for this. First, while both PAP and NT5E are capable of acting as AMPases in vitro and in vivo, these enzymes have very different functional profiles. NT5E is an exclusive 5'-monophosphatase that selectively hydrolyzes 5'-AMP with a K_m in the low micromolar range and a pH optimum somewhere between pH 7 – 8. PAP is a promiscuous nonspecific phosphomonoesterase that can degrade numerous 5'-nucleoside monophosphates with a K_m in the low millimolar range and a broad pH range (pH 3 – 8). The differences in activity at varying pH values are especially important in the nervous system, where repeated neuronal

stimulation can produce slightly acidic conditions (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 of degrading AMP to adenosine than NT5E. However, our preliminary data examining AMP antinociception in the setting of CFA-induced inflammation suggests that both PAP and NT5E are equally important in degrading AMP under these conditions (Figure 6.2). Study of AMP degradation under different conditions in our knockout strains may help clarify potentially different roles of the enzymes.

In addition to possible differences in activity under varying extracellular conditions, PAP is also capable of degrading a wide range of other substrates besides nucleotides, while NT5E is restricted to only 5'-nucleoside monophosphates. While other physiologically relevant substrates of PAP have not been clearly demonstrated, it has been suggested that the enzyme is capable of acting on cellular substrates to inhibit cell growth and replication (Lin et al., 1992; Lin et al., 1994; Lin et al., 2001; Quintero et al., 2007). Thus, PAP could play an important regulatory role in cell homeostasis, survival, or communication.

In addition, PAP can degrade the pro-nociceptive compound 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., 2004). While we have shown that soluble PAP is capable of completely degrading LPA in vitro (Figure A1.1), our data suggest PAP decreases LPA receptor signaling through changes in downstream receptor signaling rather than directly through LPA dephosphorylation (Figures A1.2, 4.15). However, LPA-degrading enzymes can significantly lower extracellular LPA levels through direct breakdown (Pilquil et al.,

2001; Xu et al., 2000), suggesting PAP may also be capable of performing this action. While the antinociceptive actions of PAP appear to be dependent on adenosine production, it is possible that in the setting of long-term elevations in LPA, PAP could debulk LPA levels and affect nociception or other LPA-induced processes.

In addition, breakdown of some forms of LPA produces the antinociceptive cannabinoid 2-arachidonylglycerol (2-AG) (Hillard, 2000; Nakane et al., 2002). Thus, in one step, PAP could degrade a pro-nociceptive compound and produce an antinociceptive one. However, more studies are needed to determine if PAP performs these reactions in the nervous system in vivo and if they are physiologically relevant. The potential roles of PAP in the nervous system may go beyond degradation of AMP, while NT5E's actions are likely to be limited in scope.

6.3) Regulation of Cellular PIP_2 by Ectonucleotidases

In elucidating the mechanism of action of PAP, we have proposed a potentially novel mode of regulation of cellular activity by ectonucleotidases. Using cellular Ca^{2+} responses as a measure of receptor activation, we have shown that PAP reduces activation of the capsaicin and noxious heat receptor TRPV1, as well as signaling through LPA, ATP, thrombin, and bradykinin receptors (Figures 4.2, 4.12, 4.15.). Further, blocking A_1R activation, blocking PLC activation, or restoring PIP_2 levels by overexpression of a PI(4,5)P kinase all reverse this PAP-mediated inhibition. This led us to propose a model whereby PAP-mediated inhibition occurs through chronic activation of A_1R and subsequent depletion of cellular PIP_2 in vitro (Figure 4.1). Importantly, we also show that inhibiting PLC activity or directly restoring PIP_2 levels in vivo blocks PAP-mediated thermal antinociception. In addition,

genetic deletion of PAP leads to increased levels of PIP_2 in DRG, while PAP injection decreases DRG PIP_2 levels.

We also have preliminary data that suggests NT5E might act through the same mechanism. While overexpression of NT5E alone leads to only small (but significant) reductions in LPA-evoked Ca^{2+} responses, this effect can be significantly increased through overexpression of both NT5E and A_1R (Figure A2.1B). Whether or not this effect is due to PIP_2 depletion has yet to be determined. However, PIP_2 levels are significantly increased in DRG from *Nt5e*$^{-/-}$ and *A*$_1$*R*$^{-/-}$ mice (Figure 5.7), suggesting loss of the ectonucleotidase increases cellular PIP_2 in vivo. The antinociceptive effects of recombinant mNT5E are dependent on A_1R activation, but whether they are due to downstream PIP_2 depletion is unknown. However, taken together, these data suggest a novel mechanism for the regulation of cell signaling by AMP-degrading ectonucleotidases, where chronic activation of A_1R by adenosine leads to low-level PLC activation and depletion of cellular PIP_2.

We should point out that activation of A_1R also leads to other downstream effects, including inhibition of adenylate cyclase, decreased cellular levels of cAMP, decreased PKA activity, and DAG-mediated activation of PKC. While these signaling pathways are important in sensitization or desensitization of nociceptors through various mechanisms, our studies show that these pathways are not involved in PAP-mediated antinociception (Figures 4.7, 4.14). Indeed, all of our data support changes in cellular PIP_2 levels rather than changes in kinase activity as the mechanism of action of PAP. However, while the antinociceptive effects of PAP clearly depend on PIP_2 depletion, we cannot rule out additional effects of PAP through modulation of these other signaling pathways.

Our model of ectonucleotidase-mediated antinociception emphasizes the importance of cellular PIP_2 levels. We propose that the "phosphoinositide tone" of the nociceptor sets its relative excitability, and that dynamic changes in PIP_2 levels allow the neural circuitry to easily adjust to abrupt changes in synaptic input (Figure 6.3). This is supported by our observations using S-hPAP injection and *Pap*$^{-/-}$ mice that basal PIP_2 levels in DRG are directly correlated with nociceptive responses, such that low PIP_2 levels at the time of chemical insult (LPA or ATP) result in the development of negligible hyperalgesia, while high PIP_2 levels at the time of insult lead to excessive hyperalgesia (Figures 6.4A, 6.4B). Further, directly increasing PIP_2 levels acutely at the time of LPA injection enhances the subsequent thermal hyperalgesia and mechanical allodynia, (Figures 4.18A, 4.18B) while decreasing PIP_2 levels through injection of PAP prior to physical nerve injury reduces subsequent hyperalgesia (Figure 4.19). Surprisingly, directly increasing PIP_2 levels in DRG and spinal cord increases thermal sensitivity in naive animals (Figures 4.11D, 4.11E), suggesting not only a role for PIP_2 in sensitized states, but also in setting the gain of nociceptors in unsensitized states. These results suggest a novel approach to the treatment of pain, namely the direct modulation of PIP_2 levels. This approach should not be entirely surprising, given that numerous ion channels known to be involved in nociception are regulated by PIP_2 (see below). The development of tools to directly and rapidly adjust PIP_2 levels in vitro and in vivo will be key in supporting this new model.

We should point out that we have yet to determine the exact location in the nervous system where PAP and NT5E are activating A_1R and depleting PIP_2. Specifically, are these effects occurring in 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? While we cannot make definitive conclusions based on our current data, we can speculate about these various possibilities. It seems unlikely that sufficient levels of PAP, NT5E, or A_1R are present in peripheral terminals to have large effects on PIP_2 levels there. However, peripheral injection of capsaicin into the hindpaw of mice produced enhanced nocifensive responses in *Pap*$^{-/-}$ mice, suggesting loss of the enzyme led to increased activity of TRPV1 in peripheral terminals (Figure 4.2F). Whether this was due to increased PIP_2 is unclear and will be difficult to test, as no good methods currently exist to measure PIP_2 in axon terminals.

Centrally, endogenous PAP and NT5E are ideally suited to act on A_1R in the spinal cord. A_1Rs are concentrated in lamina II of the dorsal spinal cord, particulatly on postsynaptic neurons that are in close contact with $IB4^+$ neurons (Schulte et al., 2003). Evidence also exists for the presence of A_1R in presynaptic terminals in lamina II from small- to medium-diameter DRG neurons (Lao et al., 2001; Li and Perl, 1994; Schulte et al., 2003). Thus, PAP and NT5E could be activating A_1R on both pre- and postsynaptic membranes to deplete cellular levels of PIP_2 in both locations. While it is difficult to measure PIP_2 levels in these regions, this could be tested using electrophysiology in spinal cord slices with direct addition or sequestration of PIP_2. Intrathecal injection of S-hPAP should not only activate A_1R in dorsal horn neurons and terminals, but also in other spinal cord regions. This includes not just other neurons, but also microglia and astrocytes that express adenosine receptors. It is likely this would also reduce PIP_2 in these other regions and cell types, but whether this reduction has physiologic effects is unclear.

6.4) PIP_2 Modulation of TRPV1

The direct modulation of TRPV1 by PIP_2 has been a controversial topic of debate for quite some time. Initial reports of a direct inhibitory role for PIP_2 by binding to TRPV1 have subsequently been refuted by evidence supporting a direct activating effect of the lipid in excised patches and other systems (see Section 1.4.2.2.1). This has led some to propose dual activating and inhibitory effects of PIP_2 at low stimulus concentrations and a simple activating effect at high stimulus concentrations (see Figure 1.4) (Rohacs et al., 2008). Numerous studies now seem to support this model, particularly the activating effect of PIP_2 on TRPV1 and the requirement of PIP_2 for the recovery of the channel from desensitization (see Figure 1.3 and Section 1.4.2.2.1).

Our data also support an activating effect of PIP_2 on TRPV1 activity. Importantly, we have been the first to directly modify PIP_2 levels in vivo through injection of PAP (to reduce PIP_2) or direct injection of PIP_2 and to show effects on thermal hyperalgesia, predominantly through TRPV1 (Figure 4.12). These in vivo results are more compelling and more biologically relevant than in vitro studies using exclusively cultured cells, where differences in cell-line characteristics, expression of regulatory proteins, expression levels, and functional properties can vary. This includes cultured DRG neurons that can change TRPV1 expression or activity as a result of the culturing process (Story et al., 2003a). While our studies will add to the bulk of the published data that supports a stimulatory role of PIP_2 on TRPV1, the debate will likely continue until direct in vivo manipulation of PIP_2 coupled with measurement of TRPV1 currents occurs.

6.5) Other Channels Potentially Affected by Ectonucleotidase-Mediated PIP_2 Modulation

A growing body of evidence shows that PIP_2 is not only important in regulating TRPV1 signaling, but also a whole host of other ion channels that are important in nociceptive signaling. This PIP_2 modulation of ion channels is very advantageous, as it 1) prevents channels from becoming active until they are incorporated into the plasma membrane, and 2) allows for dynamic regulation of channel activity following neurotransmitter-mediated activation of PLC and subsequent PIP_2 depletion (Suh and Hille, 2008). By modulating cellular levels of PIP_2, PAP or NT5E could also regulate the activity of these channels. While we have shown that a portion of PAP's thermal antinociceptive effect is due to inhibition of TRPV1, the remaining thermal effects and all of the mechanical effects could be due to changes in activity in these other PIP_2-sensitive channels. Here I will highlight several candidate channels that are involved in nociception and are known to be regulated by cellular PIP_2 levels. These channels are potential downstream targets of ectonucleotidase activity.

Voltage-gated Ca^{2+} channels (VGCCs) are expressed in almost all excitable cells and transduce electrical activity to intracellular biochemical signals at neuronal synapses (Cao, 2006). They are large, multi-protein complexes made up of a central, pore-forming $\alpha 1$ subunit surrounded by auxiliary $\alpha 2\delta$, β, and γ subunits. VGCCs are very diverse, due to different combinations of auxiliary subunits, as well as alternative splicing of the gene encoding the $\alpha 1$ subunit ($Ca_v2.2$). VGCCs are divided into two categories: 1) the high-voltage activated (HVA) channels, including L-, N-, P/Q-, and R-type Ca^{2+} channels and 2) the low-voltage activated T-type Ca^{2+} channels. Only the P/Q- and N-type channels have been definitively shown to be regulated by PIP_2 (Suh and Hille, 2008).

N-type Ca^{2+} channels are abundantly expressed in peptidergic DRG neurons (with both PAP and NT5E) and are upregulated following both nerve injury and tissue inflammation (McGivern and McDonough, 2004; Westenbroek et al., 1998; Yaksh, 2006). Blockers of N-type Ca^{2+} channels reduce hyperalgesia and allodynia in models of neuropathic and inflammatory pain, and Prialt®, the synthetic form the N-type Ca^{2+} channel blocker ω-conotoxin MVIIA, is used in humans for intractable neuropathic and cancer pain (Olivera et al., 1994; Vanegas and Schaible, 2000; Yaksh, 2006). P/Q-type Ca^{2+} channels are expressed in synaptic terminals in laminae II through VI of the dorsal horn and work with N- and R-type Ca^{2+} channels to trigger neurotransmitter release (Westenbroek et al., 1998). The effects of P/Q channels on nociception is unclear, as some studies suggest a role for the channels in inhibiting neurotransmission and antinociception, while others suggest a role in activating neurotransmission and hyperalgesia (Ebersberger et al., 2004; Luvisetto et al., 2006; Ogasawara et al., 2001; Takahashi and Momiyama, 1993; Vanegas and Schaible, 2000).

Both P/Q- and N-type Ca^{2+} channels are positively regulated by PIP_2, as the lipid delays rundown and shifts the voltage-dependence of both channels to increase activity at negative potentials (Delmas et al., 2005; Michailidis et al., 2007). Depletion of PIP_2 by ectonucleotidases would thus decrease channel activity and decrease nociception in neuropathic and inflammatory pain models. These channels are of particular interest to us, as PAP and NT5E have selective effects on thermal nociception in naïve animals, but have both thermal and mechanical antinociceptive effects in models of inflammatory and neuropathic pain. VGCCs are key in the development of mechanical allodynia following nerve injury, and decreased activity of these channels due to depletion of PIP_2 could be responsible for the

antiallodynic effects PAP and NT5E. Importantly, knockouts of the α1B gene ($Ca_v2.2$) exist, and while they have significantly attenuated thermal hyperalgesia and mechanical allodynia following peripheral nerve injury, some hypersensitivity still exists (Saegusa et al., 2002). Injection of PAP or NT5E into nerve-injured α1B knockouts could be used to test the ability of these ectonucleotidases to modulate N-type Ca^{2+} channels. In addition, many pharmacological tools are available to investigate the effects of ectonucleotidase activity on VGCCs (of all types) in vitro and in vivo (Cao, 2006).

As discussed previously, TRPM8 is a non-specific cation channel expressed in sensory neurons that is activated by moderately cold temperatures and the cooling agents menthol and icillin (See section 1.4.2.1). The channel is found in small-diameter DRG neurons and is necessary for detection of cool and some noxious cold temperatures in vivo (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Several studies have shown that TRPM8 requires PIP_2 for activity. The channel's activity runs down in response to PIP_2 depletion and is reactivated by direct application of the lipid (Liu and Qin, 2005; Rohacs et al., 2005). Activation of PLC by cell surface receptors, Ca^{2+} influx, or pharmacological activators inhibits TRPM8, as does PIP_2 depletion by a rapamycin-inducible phosphatase or high concentrations of wortmannin (Daniels et al., 2009; Liu and Qin, 2005; Rohacs et al., 2005; Varnai et al., 2006; Wang et al., 2008). PIP_2 is also involved in the Ca^{2+}-dependent desensitization of the channel, as the process is slowed by the presence of the PIP_2-synthesizing enzyme PIPK5 and is accelerated by the presence of the highly Ca^{2+} sensitive PLC isoform PLCδ1 (Daniels et al., 2009; Rohacs et al., 2005). Interestingly, the affinity of TRPM8 for PIP_2 increases in response to cold, menthol, or depolarization (Rohacs, 2009; Rohacs et al., 2005). All of these data would suggest that increases in PIP_2, such as seen in

Pap$^{-/-}$ and *Nt5e*$^{-/-}$ mice, would lead to increased icillin- or cold-induced nociceptive behaviors, while decreases in PIP_2, such as those seen following injection of PAP, would lead to decreased icillin- and cold-induced nociception. It should be noted that TRPM8 is not found in either the peptidergic or nonpeptidergic subsets of nociceptors, so the channel may not localize with PAP or NT5E. We have yet to perform any studies examining the possible effects of endogenous or exogenous PAP or NT5E on cold behaviors, so possible effects on TRPM8 are unknown.

Another TRP channel, TRPA1 is also activated by noxious cold temperatures, as well as by a long list of pungent compounds and irritants (Cortright and Szallasi, 2009). This channel is solely responsible for the algesic effects of many of these compounds, including mustard oil, formalin, and cinnamaldehyde (Bandell et al., 2004a; Bautista et al., 2006a; McNamara et al., 2007; Namer et al., 2005). TRPA1 is found predominantly in peptidergic nociceptors, but its colocalization with PAP or NT5E is unknown. Activation of the receptor produces acute pain, hyperalgesia, and neurogenic inflammation. As discussed in section 1.4.2.1, TRPA1 is also thought to be important in the detection of noxious cold, although this is still in dispute. Also in dispute is the effect of PIP_2 on the modulation of the channel. Some studies have suggested a stimulatory role for PIP_2 in regulating the channel, as the lipid activated TRPA1 in excised patches and reduced Ca^{2+}-induced desensitization, while depleting PIP_2 with wortmannin inhibited the channel (Akopian et al., 2007; Karashima et al., 2008). However, other studies have suggested an inhibitory action of PIP_2 on TRPA1, as the lipid inhibited the channel in excised or whole cell patches, and PIP_2 sequestration by poly-lysine or an anti-PIP_2 antibody activated TRPA1 in the presence of inorganic polyphosphate (Dai et al., 2007; Kim et al., 2008b). Complicating matters further, a

rapamycin-inducible PIP_2 phosphatase that depletes PIP_2 and inhibits TRPM8 neither activated nor inhibited TRPA1 (Wang et al., 2008). Thus, different effects of the lipid on the channel are seen depending on the system studied and the investigators, making it difficult to predict the effects of PAP and NT5E on TRPA1. However, this contradictory data is similar to the situation seen in the literature on TRPV1, which in our hands is activated by PIP_2. We are currently initiating projects to investigate the effects of ectonucleotidases that deplete PIP_2 on TRPA1 in vitro and in vivo. Our studies should provide some of the first in vivo evidence for the role of PIP_2 in modulating TRPA1.

As discussed previously, ATP-activated P2X receptors on neurons and glia are very important in nociceptive signaling and modulation, as well as in the development of pathologic chronic pain (See section 1.5.1.1). All homomeric P2X receptors have been shown to be activated directly by PIP_2 through direct binding of the lipid to the C-terminus of the channels (Bernier et al., 2008; Mo et al., 2009; Zhao et al., 2007). Thus, PAP and NT5E activity could decrease pro-nociceptive P2X receptor signaling through two mechanisms: 1) depletion of ATP through mass-action degradation and 2) PIP_2 depletion following activation of A_1R. Both PAP and NT5E colocalize extensively with P2X3 receptors in DRG, making them ideally suited to modulate activity of these receptors. We have not studied the effects of ectonucleotidases on P2X receptor activation, but could examine this using our knockout mice and soluble recombinant proteins in conjunction with P2X agonists and antagonists.

Finally, in addition to directly modulating channels, PIP_2 has important roles in other cellular functions, including serving as a targeting anchor for proteins that catalyze endocytosis and exocytosis, for small molecular weight GTPases, and for actin cytoskeleton components (Suh and Hille, 2008). In fact, PIP_2 plays a vital role in synaptic vesicle

trafficking and recycling in the brain, as depletion of the lipid leads to delayed recycling and defective synaptic transmission (Di Paolo et al., 2004). By depleting PIP_2, PAP and NT5E could also affect these other cellular processes in neurons, which may affect nociception.

6.6) Ectonucleotidases and the Treatment of Pain in Humans

Our studies provide tantalizing evidence that ectonucleotidases in the nervous system are important modulators of nociception. This is particularly exciting, as it provides new molecular targets for the development of pain therapies. Our animal studies show that not only can these enzymes relieve "chronic" inflammatory and neuropathic-like pain, but they can prevent the development of chemical- and nerve-induced pain in the first place. Importantly, these effects occur without the development of obvious motor side-effects, a major limiting factor in the use of adenosine-receptor agonists and inhibitors of adenosine metabolism in humans. We propose that this is likely due to catalytic restriction of the adenosine production by the enzymes. By acting on endogenous substrate (namely, locally-produced AMP), PAP and NT5E are limited in their effects to those regions where AMP levels are abundant enough to lead to significant adenosine production by the enzymes. Our data suggest that modulation of endogenous PAP and NT5E or delivery of exogenous ectonucleotidases could be important new approaches for the prevention and treatment of chronic pain.

Clearly, our studies are only the very first steps in the potential development of new therapies. Many more studies are needed before this technology could be moved into humans. For example, we have shown that injection of recombinant proteins into the intrathecal space in mice has seemingly dose-dependent, long-lasting antinociceptive effects

with minimal side-effects. However, we have not closely monitored animals for important adenosine-mediated side-effects, including subtle changes in motor function, changes in cardiac function or blood pressure, or changes in sleep patterns. Further, we have not examined if repeated delivery of the proteins leads to desensitization of the antinociceptive response (tolerance) or dependence. In addition, we have only studied the effects of the proteins in one strain of male mice (C57BL/6). Studies on females and on males of different strains could prove useful, as both sex and genetic background can affect nociception (Greenspan et al., 2007; Mogil et al., 2006). In addition, we have only studied the effects of intrathecally-delivered PAP and NT5E. While this route of delivery can be used clinically, it is not well-accepted by patients. Studies on systemically- or orally-delivered forms of the proteins would be desirable. Finally, while the models of acute and chronic nociception we use are well-accepted in the field, they are relatively poor models for pathological pain in humans. The effects of PAP and NT5E in models that more closely mimic human chronic pain syndromes (for review, see Mogil, 2009) could provide important insight into the potential for these enzymes in the treatment of human disease. Despite these challenges, we remain optimistic that ectonucleotidases have great therapeutic potential as a novel treatment for chronic pain. What could these novel therapeutic approaches entail?

Obviously, the simplest tactic for the use of ectonucleotidases in pain therapy is the delivery of exogenous protein. We have already shown that this is effective in animals and is easily accomplished through the development of human recombinant proteins. These proteins can be abundantly produced in commercially-available systems and are safe for injection into humans (Burch et al., 2004; Zucchini, 2008). The biggest obstacle for the use of recombinant proteins is the mode of delivery of the drug. Peptides cannot be efficiently

delivered as oral medications, requiring local or systemic injection to bypass the digestive process for efficacy. Further, we anticipate that systemic delivery of ectonucleotidases could produce several unwanted side-effects, including motor dysfunction, cardiac dysfunction, and hypotension. Thus, recombinant hPAP or hNT5E could be injected locally or intrathecally (Figure 6.5). While this is not ideal for the treatment of chronic conditions, local delivery could be beneficial before or after surgery, dental procedures, childbirth, or local injury, such as burns, post-herpetic neuralgia, or isolated peripheral nerve injury.

Another way to deliver exogenous ectonucleotidases would be through the delivery of viral vectors that contain the genes for PAP or NT5E (Figure 6.5). This gene therapy approach could have several advantages. First, viral vectors can be developed that specifically target nociceptors, leading to increased expression of the proteins only in these neurons. This could be accomplished by using sensory neuron-selective viruses, or gene promoters that would express PAP or NT5E only in particular neuronal populations. This approach would obviously avoid the unwanted side-effects of adenosine production systemically or even in other regions of the nervous system. Further, by using viruses that incorporate their genomes into the host cell genome, expression of the ectonucleotidase could be enhanced for long periods of time, potentially indefinitely for the lifetime of the neuron. This could be very advantageous for individuals suffering from long-term chronic pain. We have recently begun studying different viral strains and genetic promoters to identify ideal conditions for the delivery of genes specifically to DRG neurons in mice. These early studies show that acute intrathecal injection of virus can lead to specific infection and expression in DRG neurons, giving us hope for the development of this strategy. Certainly the challenges and pitfalls of gene therapy are great and this type of approach in humans is certainly far-off

into the future, but the potential for big breakthroughs in the treatment of chronic pain using this strategy is great.

In addition to delivery of exogenous PAP or NT5E, we could design strategies that attempt to enhance the activity of endogenous ectonucleotidases in the nervous system (Figure 6.5). This approach would have the advantage of increasing adenosine production only in those regions where the proteins are normally expressed in nociceptive neurons. An early attempt in our lab was made to identify potential selective activators of PAP using a high-throughput screen of 30,000 small molecules. This screen examined the ability of these molecules to affect PAP-mediated degradation of a generic fluorescent substrate. While we were successful in identifying some new potential inhibitors of PAP, we did not identify any selective activators of the enzyme.

A more exciting strategy is the use of selective pro-drugs that could be acted upon by PAP or NT5E (Figure 6.5). These are essentially versions of the A_1R-selective agonist N6-cyclopentyladenosine (CPA) that have various protecting groups that make them selectively dephosphorylated by only PAP or NT5E, resulting in the antinociceptive compound CPA. These drugs have several advantages, including the ability to modify them for easy systemic and oral delivery. This makes them more desirable for the treatment of chronic conditions, but also opens the possibility of off-site effects. This is of special concern for pro-drugs for NT5E, since the enzyme is widely expressed in many tissues. Pro-drugs specific for PAP would be highly desirable, as it has a more restricted tissue expression profile. Early studies with several of these drugs have shown promising antinociceptive effects when delivered intrathecally, systemically, and orally. However, further work is needed to examine possible side effects and pharmacological properties, such as tolerance and dependence.

Finally, an additional approach for the use of endogenous PAP or NT5E to treat pain would be to upregulate gene expression in DRG or spinal cord through chemical or genetic approaches. This tactic would be especially important in the setting of nerve injury-neuropathic pain, as both PAP and NT5E show dramatic downregulation in expression following peripheral nerve injury (Shields et al., 2003; Vadakkan et al., 2005) (Figure 5.5). This strategy would require detailed knowledge of the gene structure and identification of activators of gene expression. Unfortunately, the regulation of PAP and NT5E expression is largely unknown, making this approach difficult at this time. Future work on PAP and NT5E gene regulation could, however, make this an appealing strategy.

Clearly all of the potential therapeutic approaches outlined above provide challenging obstacles. However, these are problems worth tackling. Chronic pain is an enormous problem in the Western world, resulting not only in severe deleterious effects to the individual patient, but also significant financial burdens to society as a whole. The current treatments for chronic pain conditions are only moderately effective and have undesirable side effects. As a result, the identification and study of new targets for pain therapy, such as ectonucleotidases, provide hope for the development of new and better treatments to reduce both the immense suffering of afflicted patients and the societal ills associated with chronic pain therapy.

6.7) Other Potential Therapeutic Applications of Ectonucleotidases

Adenosine receptors are involved in numerous physiologic processes throughout the body. Numerous studies have examined the use of adenosine receptor analogs or antagonists in the modulation of receptor activity in disease states (for review, see Jacobson and Gao,

2006). Some of these compounds are in clinical trials for the treatment of specific pathological conditions. Another approach to modulation of adenosine receptors that could avoid some of the known side effects is to target ectonucleotidases. By increasing or decreasing ectonucleotidase activity globally or locally, it could be possible to affect other pathological conditions besides pain that involve adenosine receptors. Below, I highlight several processes known to involve adenosine receptor signaling that could potentially be treated through the use of some of the therapeutic approaches described in the previous section.

Sleep

Adenosine is an important player in the modulation of sleep. This is identifiable for most adults who rely on the stimulant caffeine, a well-known antagonist of A_1 and A_{2A} receptors, to get through the day. Numerous studies have elucidated the important role of adenosine in the regulation of several aspects of sleep. Specifically, local adenosine levels rise in certain brain areas during waking and decline during sleep (Huston et al., 1996; Murillo-Rodriguez et al., 2004; Porkka-Heiskanen et al., 1997). Further, this adenosine mediates the somnogenic effects of prior wakefulness and is vital in regulating the duration and depth of sleep after wakefulness (Landolt, 2008; Porkka-Heiskanen et al., 1997). Inhibition of adenosine metabolism (and thus increases in extracellular adenosine) prolongs sleep and increases the intensity and depth of non-REM sleep (Okada et al., 2003; Radek et al., 2004; Radulovacki et al., 1983). This effect is similar in magnitude and character to the effects of prolonged sleep deprivation. Early evidence suggested this effect was due to activity at A_1R (Benington et al., 1995; Schwierin et al., 1996; Thakkar et al., 2003), but $A_1R^{-/-}$ mice do not show changes in homeostatic aspects of sleep-wake regulation (Stenberg et al.,

2003). More recently, $A_{2A}R$ has been shown to play a major role in mediating these effects, as $A_{2A}R$ agonists induce non-REM sleep and $A_{2A}R^{-/-}$ mice do not show non-REM sleep rebound after sleep deprivation (Hayaishi et al., 2004; Methippara et al., 2005; Satoh et al., 1999; Urade et al., 2003). Further, $A_{2A}R$ is thought to be the main target for caffeine-induced wakefulness (Huang et al., 2005).

Endogenous NT5E likely plays an important role in these adenosine-mediated sleep effects, as it is expressed widely in the brain (Langer et al., 2008). Current data suggests it is found mostly on astrocytes and not neurons (Jacobson and Gao, 2006), but we have not tested our anti-NT5E antibody in brain to confirm this. We have not been able to identify PAP expression in the brain. Changes in sleep patterns in $Nt5e^{-/-}$ mice have not yet been studied. However, in theory, drugs that modulate NT5E or PAP activity could prove to be important in regulation of sleep. Pro-drugs, activators, or recombinant proteins could provide a promising approach for treatment of sleep maintenance insomnia. Conversely, inhibitors of NT5E could be stimulants, similar to caffeine that could be used for treatment of excessive sleep conditions (narcolepsy). Of course, limiting the usefulness of such drugs would be the potential side-effects due to off-site actions through global effects on adenosine production.

Hypoxia

Adenosine plays a vital role in the adaptation to hypoxia in a number of tissues. Tissue and plasma adenosine levels increase dramatically during hypoxic stress (Gnaiger, 2001), and this adenosine is thought to be an adaptive response to conditions of low oxygen through several different adenosine-receptor induced processes. Activation of A_1Rs helps prevent neurotoxicity (through inhibition of neuronal firing) and decreases metabolic activity

and ATP demands (Matherne et al., 1997). Activation of $A_{2A}R$ leads to vasodilation and increased ventilation (Conde et al., 2009; Jacobson and Gao, 2006; Monteiro and Ribeiro, 1987). $A_{2B}R$ activation induces vasodilation in some vascular beds (Rubino et al., 1993; Szentmiklosi et al., 1995), promotes angiogenesis (Feoktistov et al., 2004), inhibits growth of vascular smooth muscle cells (Dubey et al., 2000), and inhibits cardiac fibroblast growth (Chen et al., 2004). In addition, several studies have suggested a protective role for A_2 receptors in maintaining vascular barrier function (Weissmuller et al., 2005), which can breakdown in severe hypoxia (such as seen at high altitude), leading to cerebral or pulmonary edema. The combination of these effects through different adenosine receptors leads to a complex system of beneficial adaptations to both acute and chronic hypoxia.

An important role for endogenous NT5E in adenosinergic adaptive responses to hypoxia is supported by many findings. First, the increase in adenosine seen in the CNS in response to hypoxia is almost completely due to extracellular nucleotide degradation (Koos et al., 1997). Further, hypoxia induces upregulation of NT5E expression, through a hypoxia-inducible factor-1 (HIF-1)-mediated mechanism (Eltzschig et al., 2003; Ledoux et al., 2003; Synnestvedt et al., 2002). This increase in NT5E expression is also functionally important, as hypoxia-induced vascular leak syndromes in several organ systems (lung, heart, intestine, and kidney) are significantly accentuated following pharmacologic blockade or genetic deletion of NT5E (Thompson et al., 2004a).

Due to the importance of extracellular adenosine production in the adaptation to hypoxia, ectonucleotidase-targeting therapies could prove beneficial in individuals anticipating exposure to hypoxic conditions. Two broad strategies could be attempted. First, enhancement of endogenous NT5E activity could be used to increase extracellular adenosine

to levels at or above those seen in hypoxia. This could be accomplished either through increasing the expression of NT5E (as is seen to occur following HIF-1α pathway activation), increasing the activity of the protein using small-molecule activators, or increasing the levels of NT5E substrate present through the delivery of pro-drugs. Second, increased ectonucleotidase activity could be accomplished through the delivery of recombinant PAP or NT5E. In fact, delivery of NT5E protein isolated from rattlesnake venom reduced vascular permeability in wild-type mice at both normoxic and hypoxic conditions, reducing subsequent pulmonary edema (Thompson et al., 2004a). This suggests that delivery of ectonucleotidases prior to exposure to hypoxic conditions (such as high altitude) could prevent vascular leak. Of course, as discussed previously, systemic administration of recombinant PAP or NT5E could be limited by potential cardiac and hemodynamic side effects.

Cardiac Ischemia

Adenosine is released from cells of the cardiovascular system in response to stress or injury. This adenosine acts on receptors on various cell types to produce a significantly more ischemia-tolerant phenotype (Eltzschig et al., 2003). This effect is called ischemic preconditioning (IP), and can dramatically attenuate the size of subsequent myocardial infarction (Eckle et al., 2007c; Headrick et al., 2003). Surprisingly, the exact mechanisms and adenosine receptors involved in this cardioprotective effect are largely unknown (Headrick et al., 2003). IP leads to a dramatic increase in the expression of NT5E in the myocardium through a HIF-1-dependent mechanism, and NT5E AMPase activity is thought to be the major source of adenosine produced during IP (Eckle et al., 2007c; Eltzschig et al., 2003; Kitakaze et al., 1999). Pharmacologic inhibition or genetic deletion of NT5E

effectively eliminates the beneficial effects of IP on myocardial infarction size, highlighting an important role for the enzyme in endogenous responses to ischemia (Eckle et al., 2007c).

Given the substantial cardioprotective effects of IP, it would be desirable to potentially replicate these effects prior to severe ischemic insult in individuals with coronary artery disease. While many current studies are focusing on the use of selective adenosine receptor agonists for this purpose (Jacobson and Gao, 2006), drugs mimicking or accentuating the effects of ectonucleotidases could also be advantageous. Similar to the situation for hypoxia, two broad strategies could be imagined. First, enhancement of endogenous NT5E present in the myocardium could increase background adenosine levels to mimic those seen in IP. This could be accomplished by increasing the expression of the protein, increasing the activity of the protein using small-molecule activators, or increasing the levels of NT5E substrate through the delivery of pro-drugs. Second, delivery of exogenous recombinant PAP or NT5E could enhance baseline levels of adenosine to mimic IP. It should be noted, however, that delivery of NT5E isolated from rattlesnake venom did not produce effects similar to IP, nor did it enhance IP effects on myocardial infarct size when delivered in one model (Eckle et al., 2007c). In addition, systemic administration of these proteins could lead to significant side-effects due to adenosine receptor activation at other sites in the body.

Inflammation

Adenosine has anti-inflammatory properties primarily through A_{2A}Rs. This receptor is expressed on almost all immune cells and attenuates inflammation and reperfusion injury in several different tissues (Sitkovsky et al., 2004b). This primarily occurs through inhibition of neutrophil accumulation, inhibition of T-cell activation, inhibition of production of pro-

inflammatory cytokines, and enhancement of production of anti-inflammatory cytokines (Erdmann et al., 2005; Jacobson and Gao, 2006; Lappas et al., 2005; Sitkovsky et al., 2004a). Adenosine activity at $A_{2A}R$ is crucial in the limitation and termination of prolonged inflammation (Ohta and Sitkovsky, 2001), and $A_{2A}R$ agonists are being tested for use in several conditions, including sepsis, inflammatory bowel disease, arthritis, and reperfusion injury (Hasko and Cronstein, 2004; Odashima et al., 2005; Sullivan et al., 2004).

NT5E is expressed extensively in the immune system and may play a role in regulation of inflammation through extracellular production of adenosine. In particular, pharmacologic inhibition or genetic deletion of NT5E leads to increased neutrophil accumulation following hypoxia, suggesting adenosine production by NT5E is anti-inflammatory through prevention of neutrophil accumulation (Eltzschig et al., 2004). In addition, the anti-inflammatory effects of the drugs methotrexate and sulfasalazine (used for treatment of rheumatoid arthritis) depend on the activity of NT5E to make adenosine (Cronstein, 2005; Morabito et al., 1998). Thus, enhancement of endogenous NT5E or delivery of recombinant PAP or NT5E could have anti-inflammatory properties in different pathologic conditions, especially chronic inflammatory conditions like arthritis and inflammatory bowel disease.

Taste

Adenosine and adenine-containing compounds play important roles in taste sensation. Early studies suggested a possible direct role for adenosine in modulation of taste intensity. These studies showed increases in sensation of tastants of different quality (especially sweet and bitter) in the presence of the methylxanthines caffeine and theophylline, which are known inhibitors of A_1R and $A_{2B}R$ (Schiffman et al., 1986; Schiffman et al., 1985). This

effect could be counteracted by co-application with adenosine. However, later studies by other groups could not replicate this data, and a direct role in taste modulation for adenosine through its receptors is not known (Brosvic and Rowe, 1992; Mela, 1989). More recent studies have shown a clear role for AMP in the modulation of detection of bitter tastants. AMP directly inhibits activation of gustducin (the G-protein involved in bitter detection) in vitro and also inhibits detection of bitter compounds in animals and humans (Keast and Breslin, 2002; Keast et al., 2004; Ming et al., 1999). Interestingly, while several AMP-related compounds also inhibit gustducin activation in vitro (3'AMP, ADP, ATP, and adenosine 2'-monophosphate), others could not, including adenosine and cAMP. In addition to AMP, ATP also plays an important role in taste detection. ATP is the primary neurotransmitter released from taste cells (type II) and acts on P2X receptors on presynaptic (type III) cells to initiate taste transduction (Roper, 2007). Loss of $P2X_2$ and $P2X_3$ receptors reduces responses to umami, sweet, and bitter compounds (Finger et al., 2005).

Due to the importance of adenosine-containing compounds in taste sensation, potential roles for the modulation of these processes by ectonucleotidases exist. One ectonucleotidase, E-NTPDase2, is expressed in type I taste receptor cells (Bartel et al., 2006). This enzyme degrades ATP and could play an important role in the termination of P2X receptor signaling, thereby inhibiting/terminating taste detection. In addition to directly terminating P2X receptor activation, E-NTPDase2 could provide substrate for further breakdown to AMP, which can directly inhibit bitter sensation. Further degradation of AMP by NT5E or PAP would actually enhance bitter taste sensation, which could be an evolutionary advantage, as many toxic compounds have bitter qualities.

Preliminary studies in our lab suggest that NT5E and PAP are both expressed in tongue (data not shown), although the exact location of their expression is still unclear. Activity of these enzymes in taste buds could inhibit umami, sweet, and bitter taste detection through mass-action effects on ATP depletion, and/or could enhance bitter sensation through depletion of AMP. In addition to expression in the tongue, we have shown that both PAP and NT5E are expressed in the salivary gland (see Figures A5.1, A5.2, and A5.3). This raises the possibility that both ectonucleotidases are secreted in saliva and present in the buccal cavity. By breaking down AMP present on the tongue, these enzymes could enhance bitter taste sensation.

Inhibition of PAP or NT5E present in saliva and taste receptors could be beneficial in two ways. First, preventing breakdown of AMP to adenosine could increase local AMP concentrations, thereby inhibiting bitter taste sensation. This could be important for the development of more desirable drug formulations, which often have bitter qualities. Reducing bitterness could thus aid in patient compliance with prescribed regimens. In addition, makers of processed foods employ a number of techniques to hide bitter compounds created during processing, including addition of sweeteners, lipids and emulsifiers, carbohydrates, proteins, and flavors, all of which can affect the nutritional quality of the food (Roy, 1997). Inhibitors of ectonucleotidases could be a healthier alternative to these strategies. Blockade of AMP breakdown could also lead to an upstream increase in ATP levels in the taste bud, which could enhance taste transduction. Loss of taste sensitivity is a common problem among the elderly, and increasing taste cell signaling could be an effective strategy to increase taste sensation. Obviously, further studies are needed to determine the importance of ectonucleotidases in taste.

6.8) Figures and Tables

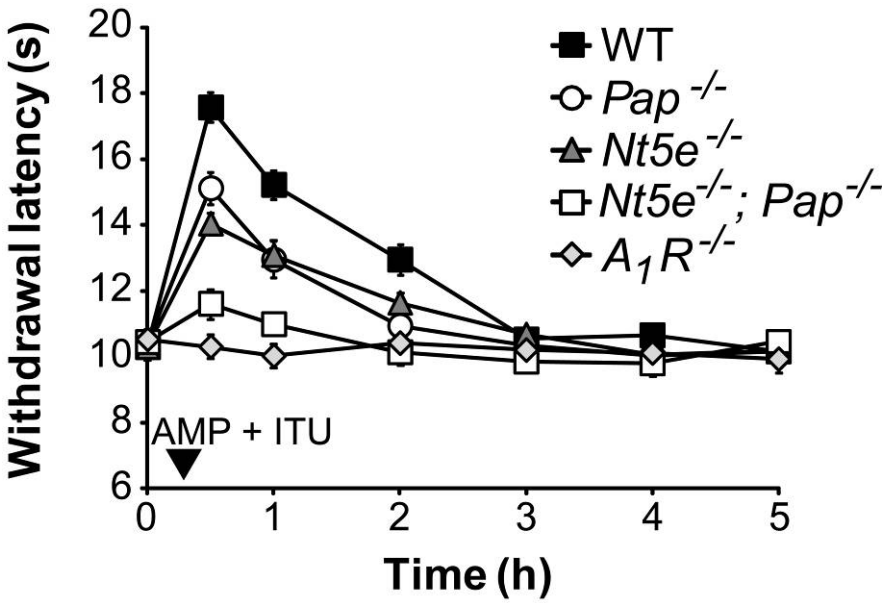

Figure 6.1. PAP and NT5E are the main AMPases in nociceptive circuits. AMPase-induced antinociception in wild-type (WT), *Pap*$^{-/-}$, *Nt5e*$^{-/-}$, *Nt5e*$^{-/-}$*; Pap*$^{-/-}$, and *A*$_1$*R*$^{-/-}$ mice. Thermal sensitivity was measured at baseline (t = 0) and after i.t. injection of AMP (200 nmol) + 5-iodotubercidin (ITU; 5 nmol). Significant increases over baseline were seen at days 1, 2, and 3 in WT (***, ***, *), *Pap*$^{-/-}$ (***, ***, *), and *Nt5e*$^{-/-}$ (***, **, *) mice. A significant increase over baseline was seen at day 1 in *Nt5e*$^{-/-}$; *Pap*$^{-/-}$ (*) mice. Paired t tests were used to compare responses at each timepoint to BL values within a given genotype (n = 8 mice per genotype). *P < 0.05, **P < 0.005, ***P < 0.0005. All data are presented as means ± s.e.m. (Back to text)

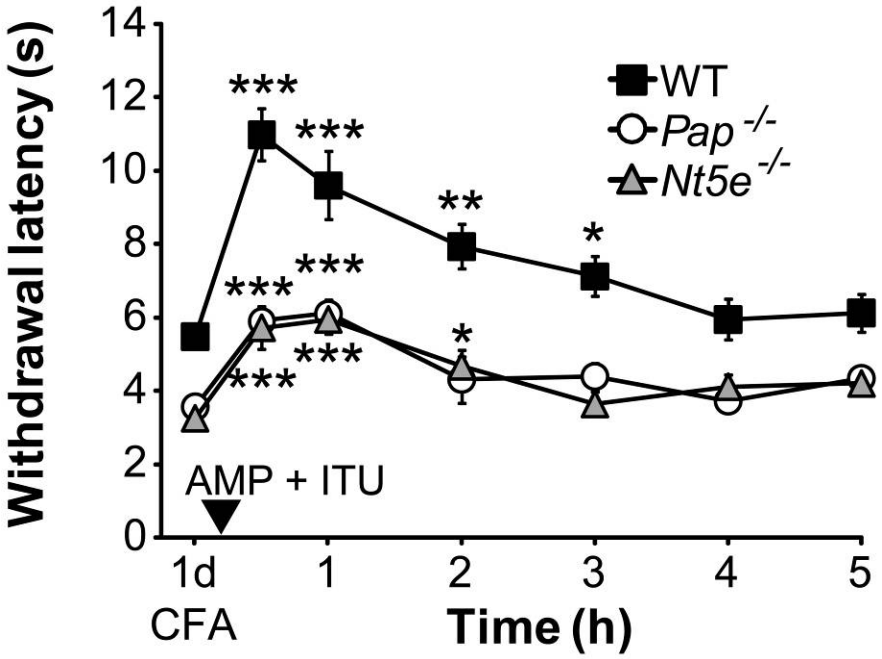

Figure 6.2. PAP and NT5E can degrade AMP during inflammation. AMPase-induced antinociception in WT, *Pap*$^{-/-}$, and *Nt5e*$^{-/-}$ mice following inflammation. Mice were tested for thermal sensitivity 1 day after injection of CFA (1d CFA) into one hindpaw. One day later, all mice were injected i.t. (arrowhead) with AMP (200 nmol) + ITU (5 nmol) and thermal sensitivity was measured for several hours after injection. Paired t tests were used to compare responses following AMP + ITU injection to the response 1 day after CFA injection in the same paw. n = 8 mice per genotype. *P < 0.05, **P < 0.005, ***P < 0.0005. All data are presented as means ± s.e.m. (Back to text)

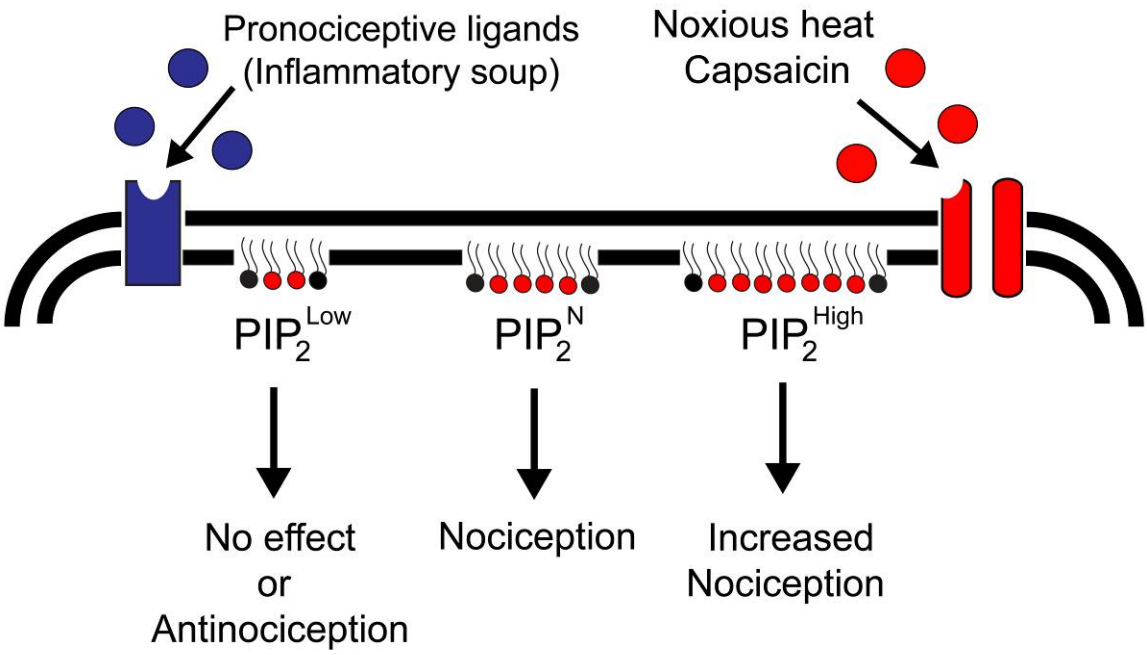

Figure 6.3. Phosphoinositide tone is a dynamic modulator of nociceptor activity. Levels of PIP_2 in the plasma membrane determine the relative excitability of nociceptive neurons in response to noxious stimuli, such as pro-nociceptive ligands and noxious heat. Activation of the nociceptor under conditions of high PIP_2 levels leads to increased nociception, while activation during a period of low PIP_2 results in little or no nociception. (Back to text)

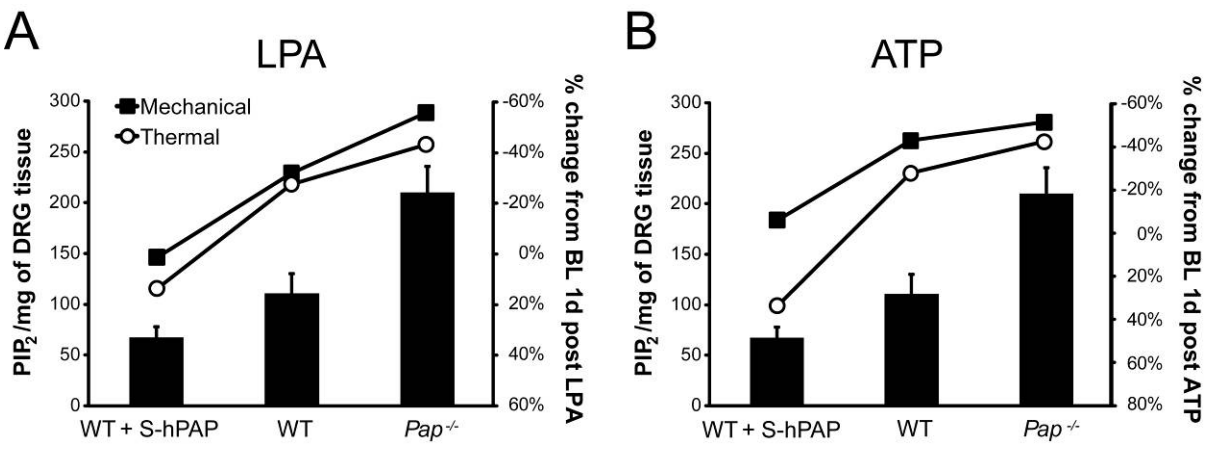

Figure 6.4. PIP_2 levels affect behavioral responses to noxious thermal and mechanical stimuli after i.t. injection of LPA or ATP. PIP_2 levels in L3-L6 DRG were quantified in wild-type (WT) mice, *Pap*$^{-/-}$ mice, or WT mice injected (i.t.) one day earlier with 250 mU hPAP (same data as shown in Figure 4.11A). Values are means ± s.e.m. n=3 mice per condition. Overlayed are the mechanical (closed squares) and thermal (open circles) sensitivities of mice one day after i.t. injection of (A) 5 nmol LPA or (B) 200 nmol ATP, expressed as percent change from baseline (BL) values. Values were calculated from data in Figures 4.16A-D (WT + hPAP, WT) and in Figures 4.17A-D (WT, *Pap*$^{-/-}$). When compared to WT mice, lower PIP_2 levels (as seen in WT + hPAP animals) correlate with no effects or antinociceptive effects on thermal and mechanical sensitivity following LPA or ATP injection, while higher PIP_2 levels (as seen in *Pap*$^{-/-}$ animals) correlate with enhanced mechanical and thermal sensitivity following LPA or ATP injection. (Back to text)

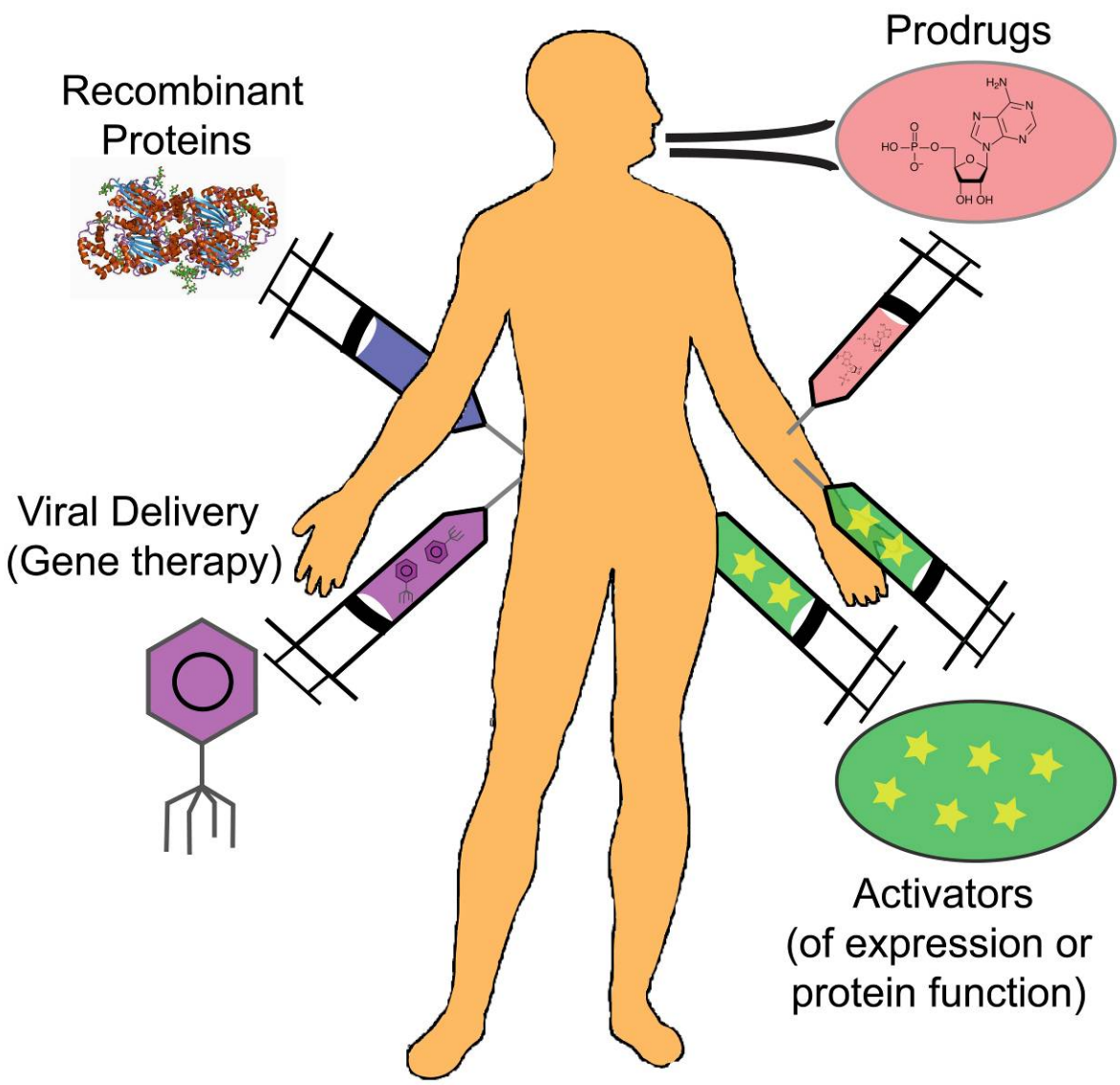

Figure 6.5. Potential therapeutic approaches targeting ectonucleotidases to treat pain. Targeting ectonucleotidases for treatment of pain could be done using recombinant versions of PAP or NT5E locally, delivery of *Pap* or *Nt5e* genes in viral vectors to the nervous system, oral or systemic administration of prodrugs acted on by endogenous PAP or NT5E to make anti-nociceptive products, or local or systemic administration of activators of ectonucleotidase gene expression or activators of PAP and NT5E enzyme activity. (See text for further details).

APPENDIX I

PAP can Directly Degrade LPA in vitro but Non-competitively Decreases LPA-evoked Ca^{2+} Responses

Summary

LPA is a common bioactive phospholipid that regulates a number of basic biological processes, including proliferation, cell differentiation, and cell survival (Brindley et al., 2002; Mills and Moolenaar, 2003; Moolenaar et al., 2004; Tigyi et al., 1994). LPA is released from platelets and neurons following tissue injury and leads to a wide range of downstream effects. These effects are mediated through a family of G-protein coupled receptors (GPCRs), three of which (LPA1, LPA3, and LPA5) are expressed in DRG neurons (Inoue et al., 2004; Lee et al., 2006; Renback *et al.*, 2000). Activation of these receptors can be measured through a variety of downstream effects, including changes in internal Ca^{2+} concentrations (Mills and Moolenaar, 2003; Moolenaar et al., 1997; Takuwa et al., 2002).

LPA is directly implicated in the modulation of nociceptor activity and pain behaviors. LPA leads to increases in intracellular Ca^{2+} levels in DRG neurons and increases in nociceptive flexor responses when injected into the hindpaw of mice (Elmes et al., 2004; Renback et al., 1999). Furthermore, intrathecal (i.t.) injection of LPA produces prolonged tactile allodynia and thermal hyperalgesia in mice, mimicking a neuropathic pain state (Inoue et al., 2004). Thus, LPA can directly affect activity of DRG neurons and lead to pathological pain states. Termination of LPA receptor signaling occurs by receptor desensitization and/or LPA degradation. PAP has been shown in one study to degrade LPA in vitro (Tanaka et al., 2004). We wished to determine if PAP could indeed degrade LPA in vitro and if this action can lead to decreased receptor activation in a cell-signaling system. By measuring Ca^{2+}

signals in response to LPA in Rat1 fibroblasts, we show that incubation of LPA with soluble PAP protein completely eliminates LPA activity at its receptors. Expression of the transmembrane (TM) form of PAP also decreases LPA-evoked calcium responses, although in a non-competitive manner. These studies suggest that while PAP is capable of directly degrading LPA, TM-PAP decreases LPA receptor signaling non-competitively downstream of receptor activation.

Methods and Results

To test the ability of PAP to degrade LPA in vitro, 0.5 units of soluble human PAP (hPAP) was incubated with 0.2 mM LPA for 0, 10, 30, or 60 min at 37ºC. Following incubation, hPAP enzyme activity was stopped by freezing the samples at -80ºC until use. Degradation of LPA was determined by examining the ability of the incubated LPA to activate receptors on Rat1 fibroblasts. To study LPA-evoked Ca^{2+} responses in whole cells, Rat1 fibroblasts were plated on glass-bottom dishes and loaded with the ratiometric calcium indicator dye Fura-2 AM (Invitrogen) (For detailed methods, see Section 4.2.3). LPA incubated with hPAP was added to the loaded cells and changes in internal calcium were monitored for several minutes. LPA that was incubated with hPAP for 0 minutes (the enzyme was frozen immediately after adding to LPA) showed a robust, transient increase in internal Ca^{2+} (Figure A1.1). Incubation of LPA with hPAP for increasing amounts of time led to decreases in LPA-evoked Ca^{2+} responses, such that after 60 min of incubation, almost all LPA activity was eliminated. This suggests PAP can directly degrade LPA and prevent it from activating its receptors.

To test if the TM form of PAP could also degrade LPA and decrease LPA-evoked signaling, Rat1 fibroblasts were transfected with mouse TM-PAP tagged with the fluorescent protein mCherry (see Section 4.2.2). LPA stimulation of TM-PAP-transfected cells led to a transient increase in intracellular calcium that was significantly lower in amplitude and duration than responses in untransfected control cells (see Figure 4.12A). We then conducted a dose-response of LPA-evoked Ca^{2+} responses in TM-PAP expressing cells versus untransfected controls. If TM-PAP is directly degrading LPA, the dose-response in these cells should be shifted to the right compared to untransfected cells. However, TM-PAP expression led to a shift in the dose-response down and to the right, suggesting a non-competitive mechanism of action (Figure A1.2A). This effect was due to the phosphatase activity of TM-PAP, as a catalytically inactive form of the protein (TM-PAP(H12A), see Section 4.2.2) did not significantly change the LPA dose-response (Figure A1.2B). Together, these results suggest that while PAP is capable of directly degrading LPA, it is most likely decreasing LPA-receptor activity in a cellular context by inhibiting some aspect of cellular signaling downstream of receptor activation.

Figures

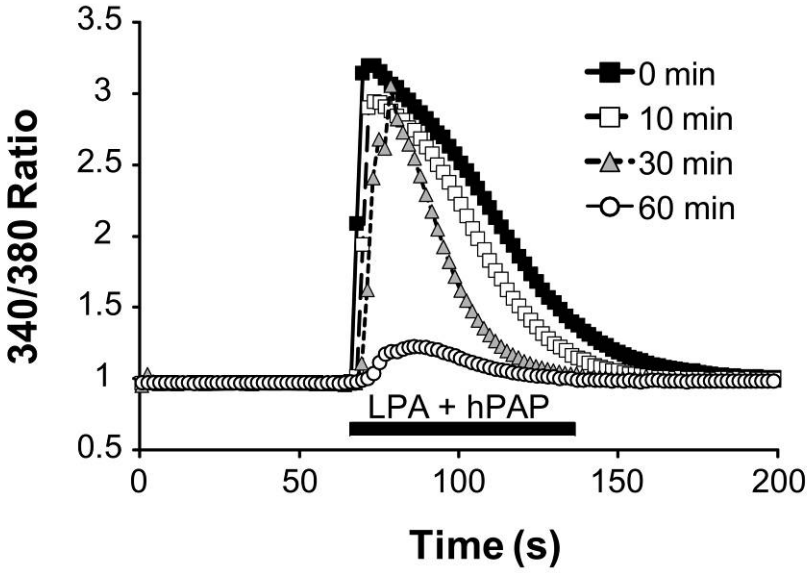

Figure A1.1. PAP can degrade LPA in vitro. LPA (0.2 mM) was incubated with soluble hPAP (0.5 U) for the indicated amounts of time and then added to Rat1 fibroblasts loaded with the calcium indicator dye Fura-2 AM. Increases in intracellular calcium are seen as increases in the 340/380 ratio. Increasing the incubation time with hPAP led to decreased LPA-evoked Ca^{2+} responses, indicating increased LPA degradation. Values are means ± s.e.m. for 50 cells per dish and curves are representative experiments for each incubation time.

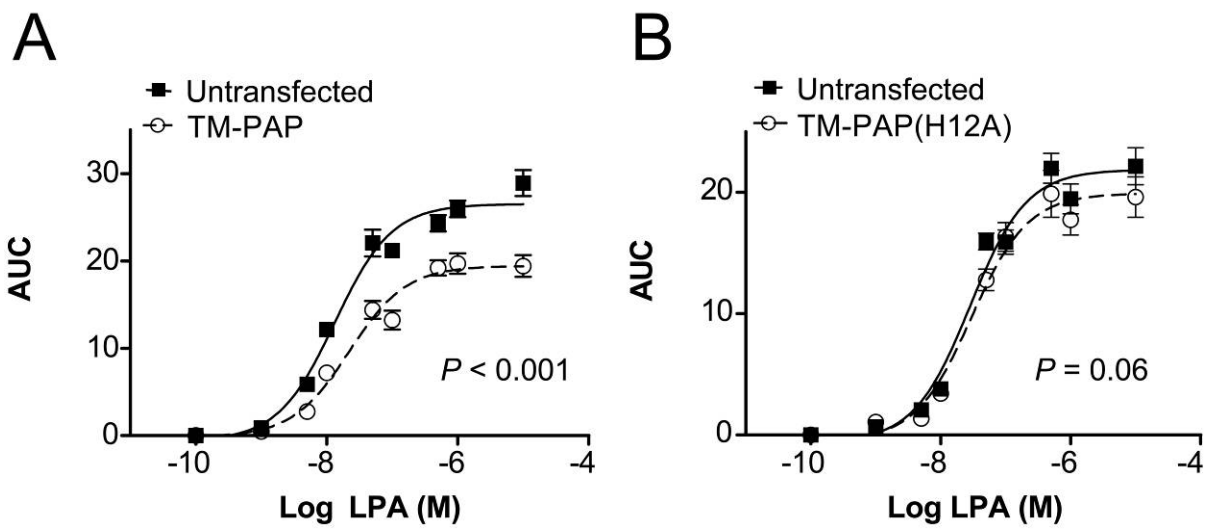

Figure A1.2. TM-PAP non-competitively decreases LPA-evoked Ca^{2+} responses. (A, B) Dose-response curves with the indicated constructs. AUC = Area under the Ca^{2+} response curve during 1 min. of LPA stimulation (see Section 4.2.3). Data are from four separate experiments. 2-way ANOVA was used to compare transfected and untransfected cells (*P* values indicated on graphs). All data are presented as means ± s.e.m.

APPENDIX II

Inhibition of LPA-evoked Ca^{2+} Responses by NT5E Following Co-Expression of A_1R

Summary

The ectonucleotidase PAP inhibits LPA-evoked Ca^{2+} responses in Rat1 fibroblasts through activation of A_1R and subsequent depletion in PIP_2. NT5E is also expressed on the cell surface and can rapidly degrade AMP to adenosine. We hypothesized that expression of NT5E in Rat1 fibroblasts would also inhibit LPA-evoked Ca^{2+} responses, perhaps through an A_1R-mediated mechanism. We made and expressed the mouse version of NT5E in Rat1 cells and stimulated them with LPA. NT5E-expressing cells showed a very small, but significant decrease in LPA-evoked Ca^{2+} responses. This NT5E-mediated inhibition could be enhanced through co-expression with the mouse A_1R receptor, suggesting NT5E can inhibit LPA receptor signaling, but only in the presence of abundant A_1R.

Methods and Results

In order to look at the activity of NT5E in vitro, we made a full-length expression construct of the mouse NT5E gene (GenBank accession #NM_011851.3) by RT-PCR amplification, using C57BL/6 mouse cDNA as template and Phusion polymerase. PCR products were cloned into pcDNA3.1 and completely sequenced. To test the effects of NT5E on LPA-evoked Ca^{2+} responses, we transfected Rat1 fibroblasts with mouse NT5E and the fluorescent protein Venus, waited 24 hours post-transfection, and then loaded the cells with Fura-2 AM. We stimulated these loaded cells with 1 μM LPA and measured the change in the 340/380 ratio inside transfected and untransfected cells in the same field of view. Cells

expressing NT5E did have a small, but significant decrease in LPA-evoked Ca^{2+} responses compared to untransfected controls (Figure A2.1B).

In order to try and increase this NT5E effect, we co-expressed mouse NT5E with a full-length expression construct of the mouse A_1-adenosine receptor (Adora1; GenBank accession #NM_001008533.2) that we generated via RT-PCR and cloned into pcDNA3.1. Surprisingly, expression of the A_1R alone caused a dose-dependent decrease in LPA-evoked Ca^{2+} responses (Figure A2.1A). However, co-expression of NT5E + A_1R led to a significantly greater reduction in LPA responses than either of the proteins alone, suggesting a synergistic effect of NT5E and A_1R (Figure A2.1B). Taken together, these data suggest that NT5E can inhibit LPA-evoked Ca^{2+} responses in vitro, perhaps through an A_1R-mediated mechanism.

Figures

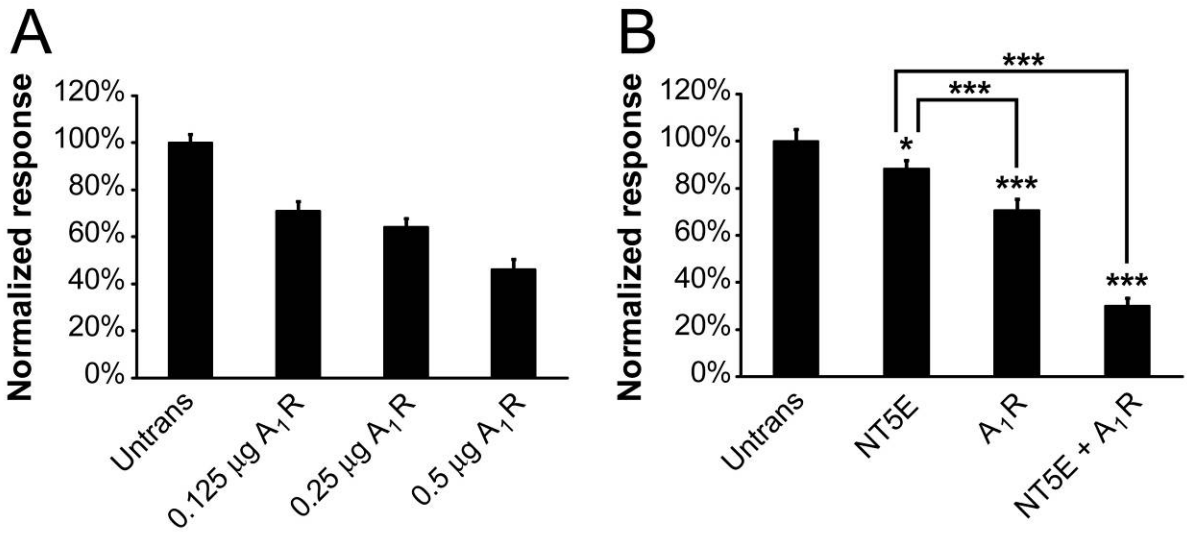

Figure A2.1. NT5E inhibits LPA-evoked Ca^{2+} responses when co-expressed with A_1R. (A) Transfection with increasing amounts of mouse A_1R inhibited LPA-evoked Ca^{2+}

responses in Rat1 fibroblasts loaded with Fura-2 AM. (B) LPA-evoked Ca^{2+} responses in Rat1 fibroblasts expressing the indicated constructs. Expression of NT5E or A_1R (0.125 μg) alone slightly inhibited responses, while co-expression of both proteins led to significantly more inhibition. For both (A) and (B), responses are the areas under the curve of the change in the 340/380 ratio over baseline normalized to the responses in untransfected control cells. For (B) t tests were used to compare untransfected cells to transfected cells, as well as cells expressing both NT5E + A_1R to cells expressing either NT5E or A_1R alone. $*P < 0.05$, $***P < 0.0005$. All data are presented as means ± s.e.m.

APPENDIX III

NT5E, but not PAP, can Rapidly Convert AMP to Adenosine and Activate $A_{2B}R$ in vitro

Summary

Both NT5E and PAP can activate A_1R, leading to decreased GPCR-induced Ca^{2+} responses. This suggests that both enzymes are capable of degrading AMP to adenosine in vitro. The A_1R-mediatd effect on cellular signaling is most likely through a low-level, chronic activation of the receptor. This does not allow us to determine if PAP or NT5E are capable of rapidly degrading AMP to adenosine and activating adenosine receptors in a cellular context. To study this, we used $A_{2B}R$, which is coupled to $G_{q/11}$ and when activated, leads to PLC activation, IP_3 production, and increases in intracellular Ca^{2+}. We transfected Rat1 fibroblasts with $A_{2B}R$ alone or with PAP and/or NT5E, loaded the cells with the calcium indicator dye Fura-2 AM, and added AMP to the cells. Cells expressing just $A_{2B}R$ showed an increase in intracellular calcium to addition of adenosine, but not in response to AMP. Cells expressing $A_{2B}R$ with NT5E or NT5E + PAP responded to AMP, suggesting NT5E can rapidly degrade AMP to adenosine. Only a small number of cells expressing PAP + $A_{2B}R$ showed a response to AMP, suggesting that PAP has limited ability to rapidly degrade AMP. These results suggest that NT5E is much more capable of rapid AMP degradation and adenosine production than PAP.

Methods and Results

To study $A_{2B}R$ activation, we transfected Rat1 fibroblasts with a full-length expression construct of the human $A_{2B}R$ tagged with the yellow fluorescent protein (YFP) (gift of Rob Tarran), and 24 hours later loaded the cells with the calcium indicator dye Fura-2 AM. We then added 1 mM adenosine (Sigma) for 5 minutes to the cells. Approximately 50% of all transfected cells showed an increase in intracellular calcium in response to adenosine (Figure A3.1A). We next added 1 mM of 5'AMP (Sigma) to cells expressing just $A_{2B}R$. Only 3.33% of cells showed any increase in intracellular Ca^{2+}, and these responses were very small in magnitude (Figure A3.1B). This suggests that these cells cannot rapidly degrade AMP to adenosine under these conditions. We next added AMP to cells co-expressing $A_{2B}R$ + mouse TM-PAP, $A_{2B}R$ + mouse NT5E, or cells expressing $A_{2B}R$ + TM-PAP + NT5E. Only 7.34% of cells expressing $A_{2B}R$ + TM-PAP showed Ca^{2+} responses to AMP, and these responses were moderate in magnitude and duration. It should be noted that significantly more $A_{2B}R$ + TM-PAP cells responded to AMP than cells expressing only $A_{2B}R$, suggesting TM-PAP is producing some adenosine under these conditions.

Strikingly, 45% of cells expressing $A_{2B}R$ + NT5E and 40% of cells expressing $A_{2B}R$ + TM-PAP + NT5E showed Ca^{2+} responses to AMP. These responses were larger in magnitude than those seen in $A_{2B}R$ + TM-PAP cells. Taken together, these results suggest that NT5E is capable of rapid AMP degradation to adenosine and subsequent adenosine receptor activation in vitro. We should note that while PAP seemed to be only weakly capable of degrading AMP to adenosine in this assay, it is possible that PAP could be making adenosine rapidly, but this adenosine is incapable of quickly activating $A_{2B}R$ before being taken up into the cells. Evidence exists that suggests some ectonucleotidases may actually have a very close association with adenosine receptors, essentially shuttling produced

adenosine directly to the receptor to maximize activation (Cunha et al., 1998). This could be the case for NT5E, while PAP may not be closely coupled to $A_{2B}R$ in this experimental setup. Further studies are needed to test this hypothesis.

Figures

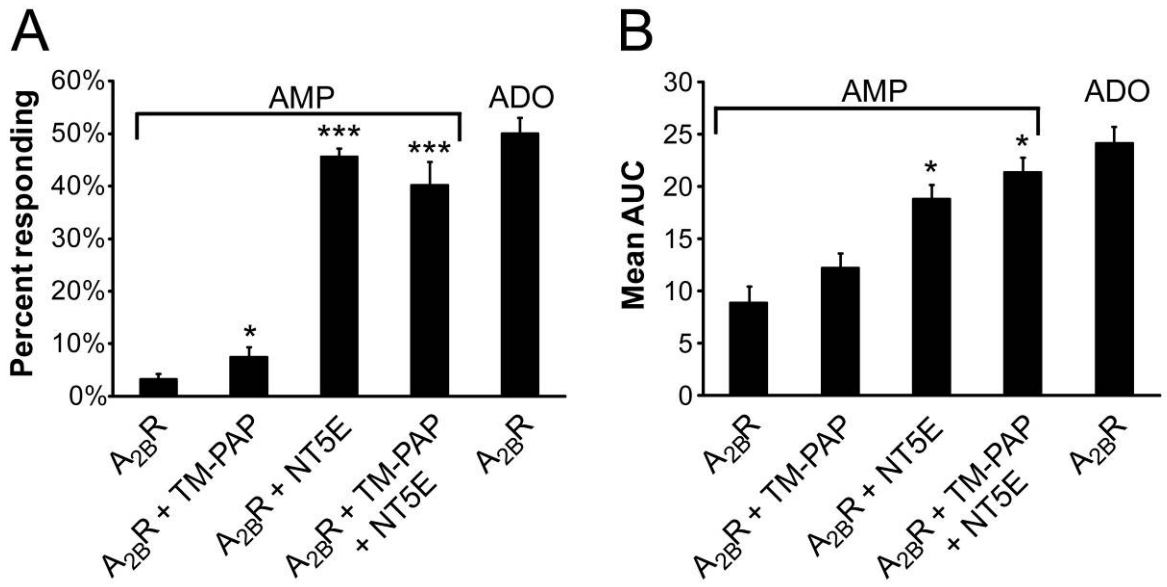

Figure A3.1. NT5E and PAP can rapidly degrade AMP to adenosine and activate $A_{2B}R$. (A) Percent of total Rat1 fibroblasts expressing the indicated constructs that showed an increase in intracellular Ca^{2+} in response to AMP (1 mM) or adenosine (ADO; 1mM). An increase in intracellular Ca^{2+} was defined as an area under the curve value > 5 over the first minute of AMP addition for a given cell. Four separate experiments were conducted for each condition. (B) The mean Ca^{2+} response seen in cells transfected with the indicated constructs that showed an increase in intracellular Ca^{2+} in response to AMP (1 mM) or adenosine (ADO; 1 mM). For (A) and (B), paired t tests were used to compare values for $A_{2B}R$

stimulated with AMP to all other conditions. *$P < 0.05$, ***$P < 0.0005$. All data are means ± s.e.m.

APPENDIX IV

Thiamine as an Antinociceptive Compound: Potential Development of TMP as an Antinociceptive Pro-drug

Summary

The B vitamins, particularly thiamine (B1), riboflavin (B2), pyridoxine (B6), and cyanocobalamin (B12) have all been implicated in proper nervous system function. Deficiencies in these vitamins lead to pathological conditions, including chronic pain (Zimmermann, 1988). Conversely, supplying pyridoxine and thiamine can relieve pain in humans associated with neuropathic disorders, carpal tunnel syndrome, rheumatoid arthritis, and premenstrual tension (Abbas and Swai, 1997; Bernstein, 1990; Bernstein and Dinesen, 1993; Jorg et al., 1988; Wyatt et al., 1999; Yxfeldt et al., 2003). B vitamins also potentiate the antinociceptive effects of nonsteroidal anti-inflammatory drugs (NSAIDs) in many painful conditions (Jurna, 1998; Kuhlwein et al., 1990; Reyes-Garcia et al., 2002; Reyes-Garcia et al., 1999; Rocha-Gonzalez et al., 2004). In addition, B vitamins also attenuate neuropathic pain syndromes in animals following peripheral nerve injury, DRG compression, or induction of diabetic neuropathy (Caram-Salas et al., 2006; Sanchez-Ramirez et al., 2006; Song et al., 2009; Wang et al., 2005). Interestingly, some studies suggest that thiamine has a selective effect on reducing nerve injury-induced thermal hyperalgesia, while not affecting mechanical allodynia (Song et al., 2009; Wang et al., 2005). Some studies suggest an immediate effect of B vitamins on acute nociception, as measured in the acetic acid writhing, formalin, and hotplate tests (Moallem et al., 2008; Reyes-Garcia et al., 2001; Sanchez-Ramirez et al., 2006). The physiologic effects responsible for this anti-nociception are

unclear, although two studies have suggested direct inhibitory actions of thiamine on DRG and spinal cord dorsal horn neurons (Fu et al., 1988; Song et al., 2009). In addition, B vitamins are important in nerve conduction, enhance antinociceptive cyclic GMP production, and play a role in reconstruction of degenerated nerves (Fujii et al., 1996; Itokawa and Cooper, 1970; Vesely, 1985).

Thiamine production in the nervous system is under the control of enzymes that breakdown thiamine phosphate esters, including thiamine triphosphatase (TTPase), thiamine pyrophosphatase (TPPase), and thiamine monophosphatase (TMPase) (Knyihar-Csillik et al., 1986; Reggiani et al., 1984; Rindi et al., 1984). We recently identified PAP as the molecular identity of TMPase, and this enzyme is the only one responsible for the breakdown of TMP to thiamine in DRG and dorsal spinal cord (Zylka et al., 2008). By degrading TMP, PAP could cause antinociception due to production of thiamine. In order to initiate studies on this possibility, we attempted to study the effects of thiamine on cellular signaling and acute nociception. We show that incubation of Rat1 fibroblasts expressing the A_1R with thiamine reduces LPA-evoked Ca^{2+}, suggesting a possible role for thiamine in reducing pro-nociceptive cell signaling. Further, we show that intrathecal injection of thiamine has antinociceptive properties in two tests of acute thermal sensitivity, and some of this effect is eliminated in $A_1R^{-/-}$ mice, suggesting a possible role of thiamine in activating A_1R signaling.

Methods and Results

In order to test the effect of systemic thiamine injection on acute thermal nociception, we first attempted to recreate published data (Moallem et al., 2008) looking at latency to nocifensive responses in the hotplate test before and after intraperitoneal (i.p.) injection of

thiamine (125 mg/kg; Sigma) dissolved in 0.9% saline. We used two groups of adult male mice – 6 C57BL/6 animals, and 5 $A_1R^{-/-}$ animals. Based on PAP's requirement for $A_1R^{-/-}$ for its antinociceptive effect and a lack of an identified thiamine receptor, we used $A_1R^{-/-}$ mice as controls to see if part of PAP's effect was due to thiamine production from TMP and activation of A_1R. WT and $A_1R^{-/-}$ mice had similar latencies to nocifensive response at baseline (defined as licking hindpaw or jumping) (Figure A4.1A). Withdrawal latencies were significantly increased over baseline 30 min after i.p. injection of thiamine in both genotypes, suggesting a thermal antinociceptive effect due to thiamine that does not depend on A_1R activation.

We also tested the ability of thiamine to affect acute thermal nociception in the radiant heat (Hargreaves) test. For this test, we injected thiamine i.p. (125 mg/kg) and measured hindpaw withdrawal latencies at 30, 60, and 120 min after thiamine injection. Thiamine caused a small, but significant increase in withdrawal latency at 30 min in WT, but not $A_1R^{-/-}$ mice (Figure A4.1B). There were no changes in withdrawal latency at the later time points. This suggests that central injection of thiamine has thermal antinociceptive effects that may depend on A_1Rs.

To further test the ability of thiamine to affect cellular signaling through A_1R, we looked at LPA-evoked calcium responses in Rat1 fibroblasts expressing A_1R with or without prior incubation with thiamine. Transfected cells were incubated with 1 mM thiamine or vehicle for 3 hours, then loaded with Fura-2 AM. LPA-evoked Ca^{2+} responses were measured as described above. As described in Appendix III (see Figure A3.1A), expression of A_1R alone caused a decrease in LPA-evoked Ca^{2+} responses. Incubation of these cells with thiamine further decreased LPA-evoked Ca^{2+} responses, suggesting thiamine may

decrease cell signaling through an A_1R-dependent process (Figure A4.2). Further studies using A_1R-selective antagonists are necessary to confirm this result. Taken together, our results suggest that thiamine has effects on acute thermal nociception that may act through activation of A_1Rs. Interesting follow-up studies could be performed examining the possible anti-nociceptive properties of TMP through degradation to thiamine by PAP. If this is the case, TMP could be used as a selective pro-drug for activation by PAP for treatment of chronic pain that could avoid the side-effects of AMP analogs.

Figures

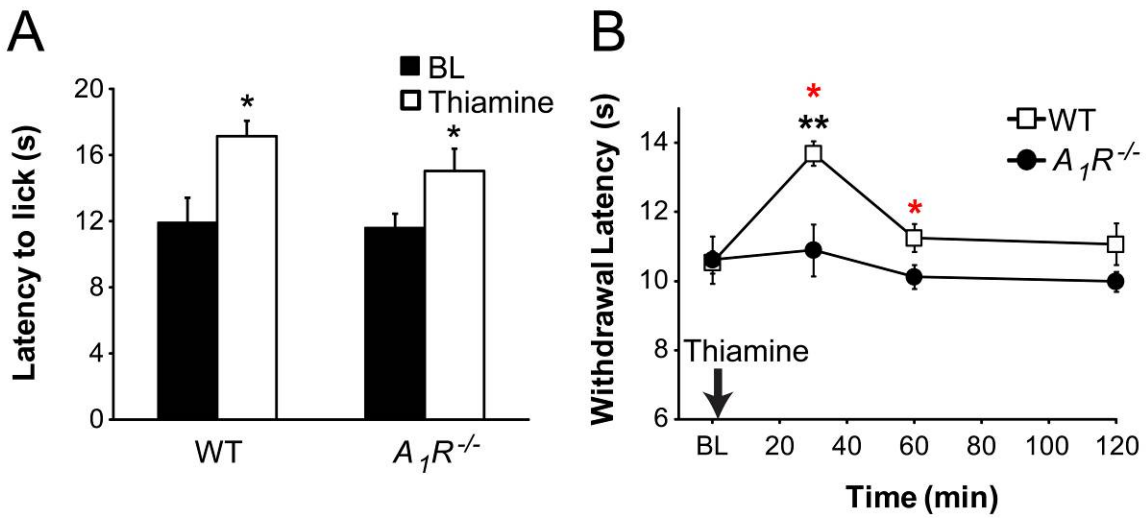

Figure A4.1. Thiamine reduces acute thermal nociception in vivo. (A) Nocifensive response to noxious heat stimulus (52ºC) on hotplate in wild-type (WT; n = 6) and $A_1R^{-/-}$ (n = 5) mice. Responses were taken at baseline (BL) on two consecutive days, followed by two consecutive days where responses were taken 30 min after i.p. injection of thiamine (125 mg/kg). A positive response was defined as licking of the hindpaws or jumping. (B) Withdrawal latency to noxious thermal stimulus (radiant light source) in WT (n = 4) and $A_1R^{-/-}$ (n = 6) mice at baseline (BL) and after i.p. injection of thiamine (125 mg/kg; arrow). For

(A) paired t tests were used to compare responses at baseline to responses at 30 min within each genotype. For (B) paired t tests were used to compare responses at BL to responses at later timepoints within each genotype (black asterisk) and to compare responses between genotypes at each timepoint (red asterisks). *$P < 0.05$, **$P < 0.005$. All data are means ± s.e.m.

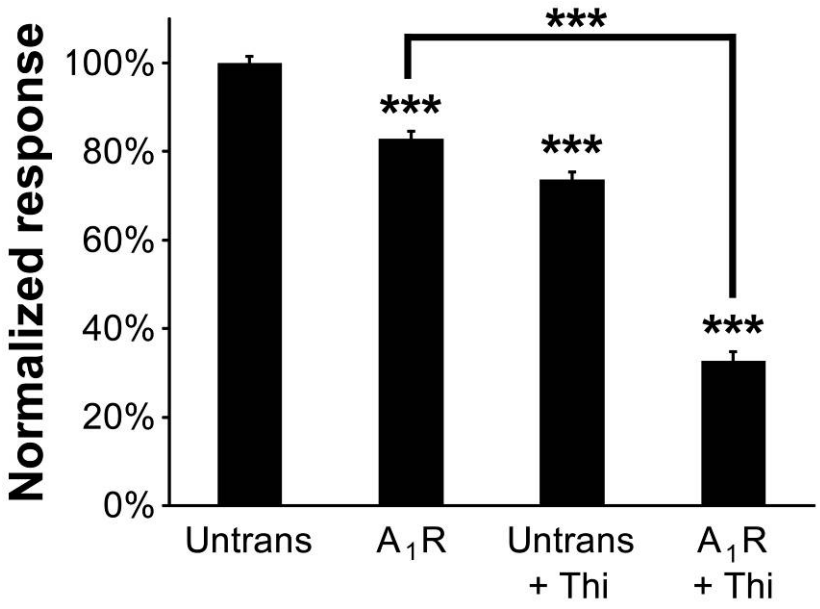

Figure A4.2. Thiamine reduces LPA-evoked Ca^{2+} responses in Rat1 fibroblasts. LPA (100 nM)-evoked Ca^{2+} responses in Rat1 fibroblasts transfected with the indicated constructs. Some cells were incubated for three hours with 1 mM thiamine (Thi) prior to LPA addition. Responses were normalized to untransfected controls. n = 180 – 240 per condition. Paired t tests were used to compare responses in untransfected cells to all other conditions and to compare A_1R to A_1R + Thiamine. ***$P < 0.0005$. All data are presented as means ± s.e.m.

APPENDIX V

Expression of PAP and NT5E in Salivary Glands of Mice

Summary

Nucleotide signaling is important in the regulation of salivary gland function. Both P2X ($P2X_4$ and $P2X_7$) and P2Y ($P2Y_1$ and $P2Y_2$) receptors are present in salivary glands and are involved in numerous processes, including chloride and sodium ion transport, secretion, response to glandular damage, and mediation of inflammation or cell death (Ahn et al., 2000; Gibbons et al., 2001; Lee et al., 1997; Turner and Camden, 1990; Zeng et al., 1997). In addition, ATP and its degradation products are secreted into saliva that eventually resides in the buccal cavity. Here, AMP and adenosine can modulate taste sensation through actions at taste buds (Keast and Breslin, 2002; Keast et al., 2004; Ming et al., 1999). ATP can be released in salivary glands from parasympathetic nerve terminals (Tenneti et al., 1998), and can also be released from salivary ductal cells in response to shear stress and distension due to salivary flow (Ralevic and Burnstock, 1998). The actions of ATP at its receptors are terminated through degradation of ATP to adenosine. Ectonucleotidases must be present in the salivary gland to mediate this process. Immunohistochemistry suggests the expression of E-NTPDase1 and E-NTPDase 2 in many different salivary gland cells types (Kittel et al., 2004), while more recent biochemical studies suggest E-NTPDase 3 may be present (Henz et al., 2006). Biochemical studies have also detected AMPase activity that was attributed to NT5E (Henz et al., 2006). However, recent evidence suggests that PAP is also expressed in mouse salivary gland and could also be responsible for some of the AMPase activity (Quintero et al., 2007).

Using enzyme histochemistry and immunohistochemistry in knockout animals, we attempted to detect the expression of NT5E and PAP in mouse salivary gland. We were able to show expression and activity of both PAP and NT5E in the salivary gland, suggesting these enzymes could play important roles in the modulation of glandular function, as well as in modulation of taste perception.

Methods and Results

In order to examine AMPase and TMPase activity in mouse salivary gland, whole salivary glands were harvested from WT, *Pap*$^{-/-}$, *Nt5e*$^{-/-}$, and *Nt5e*$^{-/-}$;*Pap*$^{-/-}$ mice, fixed for 3 hours in 4% PFA-PBS, washed in 30% sucrose, sectioned, and mounted onto Permafrost slides. AMPase and TMPase activity was performed as described previously (Zylka et al., 2008), using 5 mM of substrate at either pH 5.6 or 7.0. AMPase staining was very intense in the parotid gland of WT mouse, in acini, ducts, and connective tissue at both pH 5.6 and 7 (Figures A5.1A, A5.1B). In *Pap*$^{-/-}$ mice, staining was similar in pattern, but reduced in magnitude compared to WT, suggesting PAP is responsible for some of the AMPase activity in the WT gland (Figures A5.1C, A5.1D). In *Nt5e*$^{-/-}$, staining was diminished in the connective tissue of the gland, but still strong in the acini and ducts at pH 5.6 (Figure A5.1E). Staining at pH 7.0 was strikingly diminished in all regions, suggesting NT5E is responsible for the bulk of the AMPase activity in the WT gland (Figure A5.1F). In *Nt5e*$^{-/-}$;*Pap*$^{-/-}$ mice, staining was greatly reduced in all regions at pH 5.6 and was completely absent at pH 7.0 (Figures A5.1G, A5.1H). This suggests that PAP and NT5E are responsible for most, if not all AMPase activity in the gland.

Since we have previously shown that PAP is entirely responsible for TMPase activity in the spinal cord and DRG, we also looked at TMPase staining in the parotid gland at pH 5.6 and 7.0. TMPase staining was eliminated in both *Pap*$^{-/-}$ and *Nt5e*$^{-/-}$;*Pap*$^{-/-}$ mice at both pHs, while it was similar to wild-type in *Nt5e*$^{-/-}$ tissue, suggesting that PAP is expressed and functional in mouse salivary gland (Figures A5.2A-A5.2H).

In order to test for expression of NT5E protein, we used immunofluorescence to label salivary glands from WT, *Pap*$^{-/-}$, and *Nt5e*$^{-/-}$ mice using a commercially available anti-NT5E mouse antibody. In WT and *Pap*$^{-/-}$ mice, staining was intense in all regions of the salivary glands, similar to the AMPase activity in *Pap*$^{-/-}$ animals (Figures A5.3A, A5.3B). In *Nt5e*$^{-/-}$ tissue, staining was nearly completely eliminated (Figure A5.3C). The only residual staining appeared to be in some ducts suggesting this staining was due to non-specific binding of the antibody in these regions. These studies confirmed the presence of NT5E protein in mouse salivary gland. We also attempted to label mouse salivary gland with anti-PAP mouse antibody, but saw similar staining patterns in all genotypes tested, suggesting significant background staining in these tissues with this antibody (data not shown). Taken together, our studies confirm that both NT5E and PAP are expressed and are functional in mouse salivary gland. Their roles in salivary gland function remain to be determined.

Figures

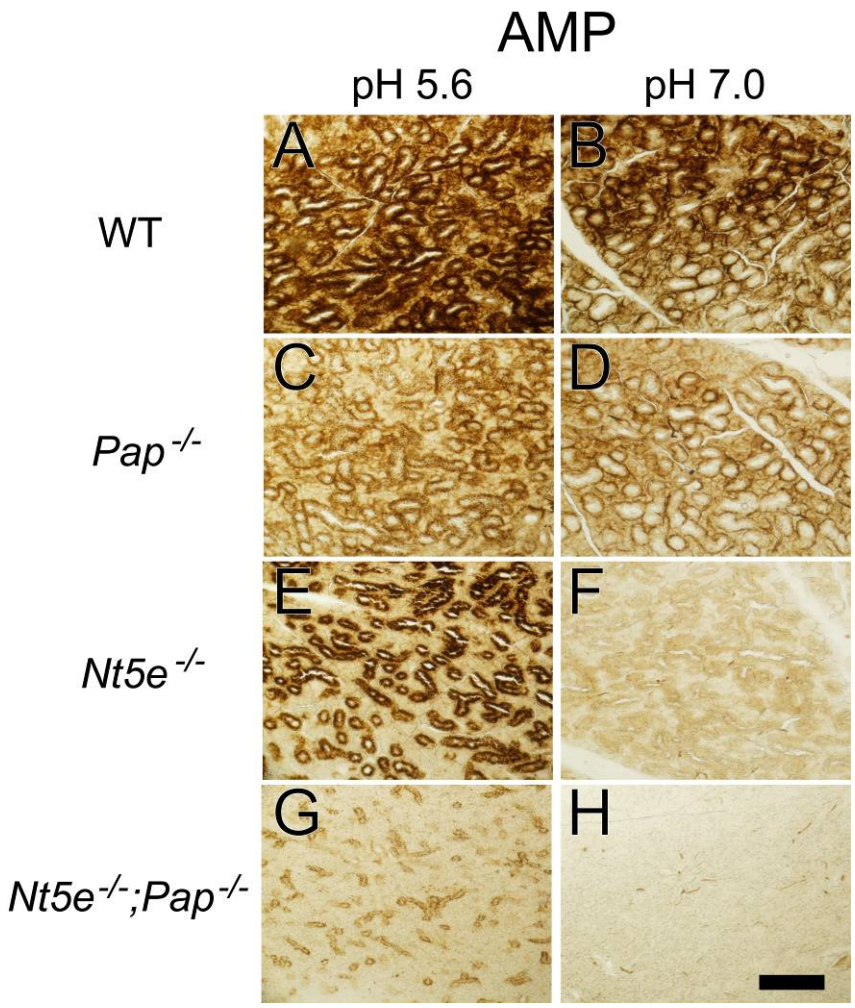

Figure A5.1. AMPase activity in mouse salivary gland. (A-H) Parotid gland from (A, B) wild-type (WT), (C, D) *Pap*$^{-/-}$, (E, F) *Nt5e*$^{-/-}$, and (G, H) *Nt5e*$^{-/-}$;*Pap*$^{-/-}$ mice stained with AMP histochemistry. AMP substrate concentration was 5 mM at the indicated pH. Scale bar is 50 μm for all images.

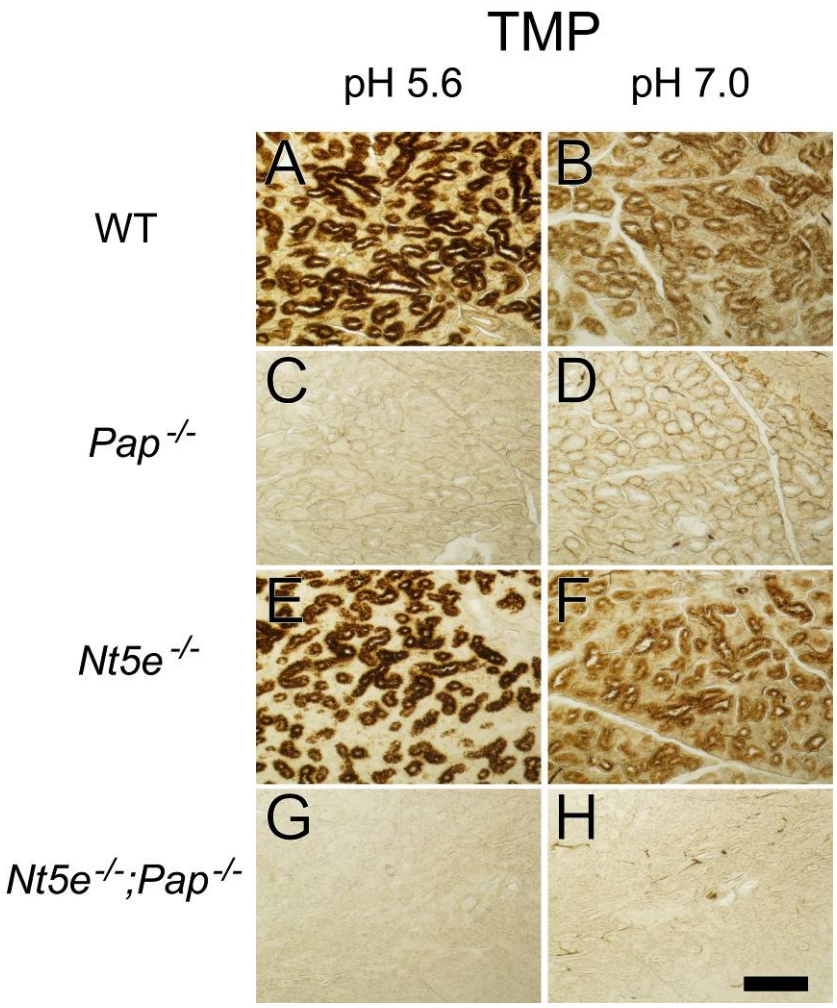

Figure A5.2. TMPase activity in mouse salivary gland. (A-H) Parotid gland from (A, B) wild-type (WT), (C, D) *Pap*$^{-/-}$, (E, F) *Nt5e*$^{-/-}$, and (G, H) *Nt5e*$^{-/-}$;*Pap*$^{-/-}$ mice stained with TMP histochemistry. TMP substrate concentration was 5 mM at the indicated pH. Scale bar is 50 μm for all images.

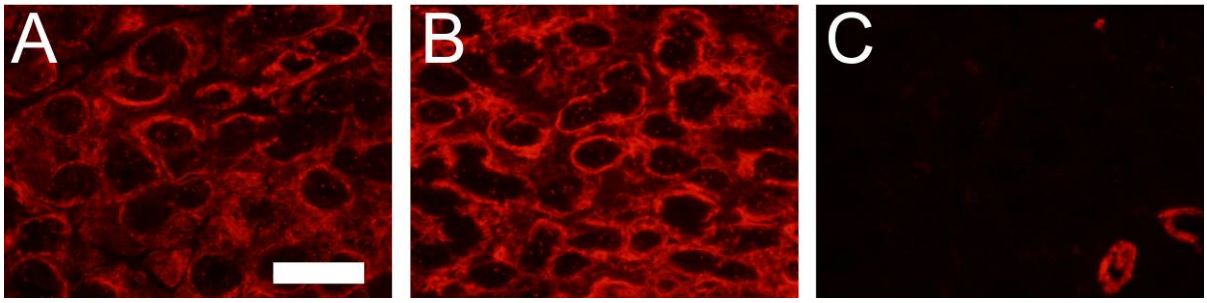

Figure A5.3. NT5E protein is expressed in mouse salivary gland. (A-C) Parotid gland from (A) wild-type, (B) *Pap*$^{-/-}$, and (C) *Nt5e*$^{-/-}$ mice labeled with anti-NT5E antibody. Scale bar is 50 μm for all images.

APPENDIX VI

Risk Factors and Characteristics of HIV Painful Neuropathy in a South African Population

Summary

Nowhere has the effects of HIV been felt on a grander scale than in the country of South Africa. Poverty, social instability, restricted access to medical care, and political indifference have combined to produce an epidemic in which over 5.7 million South Africans (18.1% of the population between 15-49) are infected (UNAIDS, 2008). The magnitude of the epidemic has had profound effects on South African society, leading to a dramatic reduction in life expectancy, an increase in deaths among young adults, an increase in child orphans (over 1.7 million "AIDS orphans"), a rise in unemployment, a stifling of the national economy, and a rise in social unrest (Booysen et al., 2003; SAIR, 2009; SSA, 2008). Social stigma and AIDS denialism amongst government officials have led to a delay in treatment of HIV-infected individuals and has also contributed to the magnitude of the epidemic, as it is estimated only 32% of those in need of treatment are currently receiving antiretroviral medications (WHO, 2008). However, the new South African government has pledged to increase the number of HIV-infected individuals receiving treatment, with a goal to provide treatment to 80% of individuals who need it by 2011 (Muir and Bass, 2009).

Peripheral sensory neuropathies (PSNs) are the most common neurological complication of HIV infection (Luciano et al., 2003; Wulff et al., 2000). PSNs result from damage to peripheral nerves that cuase slowly progressive numbness and paresthesias with burning sensations in the extremeties (Gonzalez-Duarte et al., 2007). Some patients develop

severe pain, but up to 30% are asymptomatic (Morgello et al., 2004; Schifitto et al., 2002; Schifitto et al., 2005). All individuals with PSNs are at high risk of damage to peripheral structures (hands and feet) and more serious bodily injury as a result of the progressive numbness in these regions. In addition, patients with HIV-associated sensory neuropathy have significantly lower scores in measures of quality of life, especially in perceived physical functioning, level of pain, energy level, and perceived overall health (Pandya et al., 2005).

There are two broad classifications of HIV-associated sensory neuropathy. Distal sensory polyneuropathy (DSP) is a primary complication of advanced HIV disease, seen in greater than 30% of patients with AIDS (de la Monte et al., 1988; Norton et al., 1996). The pathogenesis of DSP is unclear, but is characterized by prominent small-diameter axonal loss likely due to inflammation and is associated with low CD4 counts and elevated HIV plasma viral loads (Pardo et al., 2001; Simpson et al., 2002; Simpson et al., 1998; Tagliati et al., 1999). Antiretroviral toxic neuropathy (ATN) is a second type of HIV-associated neuropathy that is due to direct toxic effects of certain types of antiretroviral medications used to treat HIV infection. ATN is particularly associated with high doses of nucleoside reverse transcriptase inhibitors (NRTIs), including zalcitabine (ddC), didanosine (ddI), and stavudine (d4T) (Browne et al., 1993; Lambert et al., 1990; Yarchoan et al., 1988). These drugs could cause neuronal damage through mitochondrial dysfunction (Gonzalez-Duarte et al., 2007). Rates of HIV-associated neuropathies are increasing, with prevalence over 50% in some studies, and this is thought likely due in part to the development of ATN in antiretroviral-treated individuals (Morgello et al., 2004; Watters et al., 2004). However, definitive studies on the risks of antiretrovirals for development of ATN have not been performed.

Antiretroviral therapy (ART) in South Africa consists of one of two NRTIs – d4T or zidovudine (AZT) – together with lamivudine (3TC) and a non-NRTI – either nevirapine or efavirenz (Wilson et al., 2002). While the dose of d4T has been lowered recently, there is some concern that use of the drug in a population that is already at risk for polyneuropathy due to the high prevalence of malnutrition, alcohol use, and prio anti-tuberculosis therapy could lead to even higher levels of ATN. In fact, a recent cross-sectional study in a South African HIV-infected population showed that the frequency of HIV-associated sensory neuropathy increases significantly from 23% prior to initiation of ART to 40% in ART-exposed subjects (Maritz et al., 2009). In addition, a history of prior *Mycobacterium tuberculosis* (TB) infection was an additional risk factor for HIV-associated sensory neuropathy, irrespective of ART treatment status. This is especially troublesome, as South Africa has one of the highest incidence rates of TB in the world. This study seems to suggest that ART and TB treatment are significant risk factors for the development of HIV-associated sensory neuropathy. However, this study is limited by its cross-sectional design. A more rigorous, longitudinal study could more directly study the effects of ART, TB treatment, and other risk factors for the development of HIV-associated sensory neuropathy. It is exactly such a study I will be assissting in conducting.

Methods

A longitudinal study has been launched under the direction of Drs. Jeannine Heckmann (University of Cape Town), Michael Benatar (Emory University), and Taylor Harrison (Emory University) on site at the University of Cape Town Medical School and the Crossroads Community Health Center (CCHC) within the city of Cape Town, South Africa.

The CCHC provides all HIV care to approximately 5000 HIV-infected individuals in the Crossroads community. The basic study design will be similar to that of a recently completed cross-sectional study carried out in the same clinic (Maritz et al., 2009). Subjects will be included if they meet the following criteria: (1) age ≥ 18; (2) HIV infection documented by ELISA and confirmed by Western Blot; (3) No prior history of antiretroviral therapy; and (4) Patient or patient's legal next of kin understands the purpose and requirements of the study and has provided Informed Consent. Patients will be excluded on the basis of the following: (1) Cognitive impairment which, at the discretion of the site investigator is felt to preclude cooperation with the study protocol; (2) signs and symptoms suggestive of either radiculopathy or myelopathy that, in the opinion of the investigator, complicates the diagnosis of neuropathy; and (3) family history of neuropathy.

A total of three study visits will occur: a baseline visit at study entry and two follow-up evaluations at 6-month intervals. Evaluations at the baseline visit will include anthropometric data (height, weight, heart rate, blood pressure, etc.), completion of dietary, nutritional, and lifestyle questionnaires, detailed collection of past medical and social history (including the frequency and quantity of alcohol consumption, prior history of tuberculosis with documentation of regimen and treatment duration), as well as a general physical examination. Laboratory screening for diabetes through fasting blood glucose and a 2-hour oral glucose tolerance test will be performed. Subjects will also have a complete blood count, CD4+ count, HIV viral load, RPR, hepatitis B and C serology, albumin, ALT, lipids, lactate, B12, insulin level, and TSH measured.

A brief (< 30 minutes) examination of peripheral nervous system function will focus attention to extremity muscle strength, bulk, and tone, as well as to assessment of the

following primary sensory modalities: pinprick, vibratory thresholds, and proprioception. Pinprick sensitivity will assess for a symmetric, distal-to-proximal gradient of sensory loss. Vibratory thresholds measured at the distal interphalangeal joint at the first toe will be deemed abnormal if the subject is unable to report sensation beyond 10 seconds after a maximal strike of a 128-Hz tuning fork. Proprioception will be assessed at the distal phalynx of the great toe. Muscle stretch reflexes will be assessed to determine either the absence or side-to-side asymmetry of ankle jerks, or the presence of a distal-to-proximal gradient of impaired reflex elicitation. Data will be recorded in both the Brief Peripheral Neuropathy Screen (BPNS) (Cherry et al., 2005) and a modified version of the reduced Total Neuropathy Score (TNSr) (Cornblath et al., 1999). These are both well-accepted and standard means of measuring neuropathy in the field. The BPNS assesses three sensory domains (pain, numbness, and "pins and needles" or parasthesias) graded on a 10 cm visual analogue scale (VAS) as well as two examination domains consisting of vibratory sensation and reflexes. The TNSr incorporates neuropathic symptoms and clinical examination of pinprick and vibratory sensation (as described above), muscle stretch reflexes and strength. For this study, we will exclude assessment of autonomic symptoms, electrophysiology, and quantitative sensory testing. Local ethics committee approval has been secured and both neuropathy assessment tools have been translated into Xhosa. Criteria for neuropathy diagnosis, classification, and methods of grading severity are detailed in the section entitled "Outcome Measures."

Follow-up evaluations at 6 and 12 months will include the neuropathy assessment with completion of the BPNS and the TNSr as well as the following laboratory measures:

complete blood count, CD4 count, HIV viral load, glucose tolerance test, insulin level, ALT, albumin, lipids, and serum lactate.

All procedures have been approved by the University of Cape Town research ethics committee. All participants will provide written informed consent.

I will be involved in the process by performing the clinical neuropathic evaluations; these will include a brief pain symptom visual analogue scale and a brief peripheral neuropathy assessment. I will be trained and observed by study personnel during this time. I will also observe other aspects of the study. Clearly this process will not be completed at the time of submission of this document. This study will give solid evidence on the risk factors for the development of HIV-associated neuropathy in this population, with particular interest in the dangers of ART use and TB therapy. Findings from the study could prove useful in designing future treatment paradigms that limit the development of HIV-associated neuropathies.

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