CHARACTERIZATION OF RECOMBINANT MOUSE ECTO-5'-NUCLEOTIDASE

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ABSTRACT

Meagen Kathleen Voss- Characterization of recombinant mouse ecto-5'-nucleotidase

(Under the Direction of Mark. J. Zylka, PhD)

Chronic pain is the most common medical complaint in the United States and the number of Americans affected by chronic pain is growing. Effectiveness of available treatments varies greatly and many have intolerable side effects. To address this need for pain treatments, more effective therapies are needed. Recently, our lab discovered an ectonucleotidase in the central nervous system (CNS) called Prostatic Acid Phosphatase (PAP). Spinal injections of PAP into live mice produced antinociceptive effects. The following thesis describes the properties of NT5E, an ectonucleotidase that is colocalized with PAP in the CNS. We have generated a recombinant mouse NT5E protein (mNT5E) and have shown that the protein is pure, catalytically active and capable of reducing nociceptive sensitivity in two animal models of chronic pain. We also show that mNT5E acts through A_1-type adenosine receptors (A_1R). This study provides an additional target for developing pain treatments and a new tool for studying purinergic signaling.

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PREFACE

Portions of this work have been previously published:

CHAPTER 2: Sowa N., Voss M., Zylka M. (2010). “Recombinant ecto-5’-nucleotidase (CD73) has long-lasting antinociceptive effects that are dependent on adenosine A_1- receptor activation” *Molecular Pain*, in press

CHAPTER 3: Sowa N., Voss M., Zylka M. (2010). “Recombinant ecto-5’-nucleotidase (CD73) has long-lasting antinociceptive effects that are dependent on adenosine A_1- receptor activation” *Molecular Pain*, in press

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α,β-me-ADP	α,β-methylene-adenosine diphosphate
A_1R	A_1 Adenosine Receptor
AMP	Adenosine 5'-monophosphate
CFA	Complete Freunds Adjuvant
CNS	Central Nervous System
CPA	N^6-cyclopentyladenosine
ENTPD	Ectonucleoside triphosphate diphosphohydrolase
ENPP	Ectonucleotide pyrophosphatase/phosphodiesterase
FRAP	Fluoride resistant acid phosphatase
mNT5E	Mouse ecto-5'-nucleotidase
mPAP	Mouse prostatic acid phosphtase
NT5E	Ecto-5'-nucleotidase, also known as CD73
P2X	Ion-gated purinergic receptors
P2Y	G protein-coupled purinergic receptors
PAP	Prostatic Acid Phosphatase, also known as FRAP
PNS	Peripheral Nervous System
SNI	Spared Nerve Injury
TMP	Thiamine monophosphate
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate

CHAPTER ONE

BACKGROUND AND SIGNIFICANCE

1-1 Chronic Pain: A Growing National Problem

Pain is the number one health care problem faced by modern medicine. According to the American Pain Foundation, more Americans suffer from chronic pain than heart disease, cancer and diabetes combined (Foundation, 2009). Despite the high incidence of these conditions, patients suffering from chronic pain are vastly undertreated. Part of this trend can be ascribed to treatment bias on the part of medical professionals. At the same time, there is an understandable restraint in prescribing potent analgesics. Many of these drugs are accompanied by detrimental side effects that severely affect patients' quality of life. Addiction to analgesic medications, in particular, is a significant problem that is difficult to treat. Most potent analgesics will also only provide short term relief as many patients develop a tolerance to them over time.

This lack of effective treatments for chronic pain is a critical national health problem that severely impacts productivity in the workplace and continues to deplete vital financial resources. According to a study conducted by the National Institutes of Health, chronic pain treatment has an estimated cost of $100 billion per year, including cost of treatment, lost income and lost productivity (NIH, 1998). A more recent survey conducted by Harris Interactive and the National Pain Foundation in

2006 revealed that persistent chronic pain has increased by 40% in the workplace, suggesting that the incidence of pain will continue to grow unless effective treatments are implemented.

In answer to this disturbing trend, we and other scientists are investigating the molecular roots of pain; hoping that by understanding more about the pain process itself, we can develop more effective treatments. Our specific focus is on molecules that are involved in endogenous pain mechanisms that can potentially be manipulated to relieve pain. The following dissertation describes how we uncovered one of these molecules—ecto-5'-nucleotidase (NT5E)—through a powerful combination of molecular genetics and behavioral neuroscience. We hope that NT5E, and other molecules we uncover in the future, will lead to new treatments to help curb this burgeoning epidemic of pain.

1-2 How the Body Senses Pain: The Nociceptive Nervous System

Strictly defined, pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Taxonomy, 1994)". Since emotional experience cannot be interpreted beyond reasonable doubt in animals, pain is a phenomenon that is unique to humans. Nevertheless, all mammals, as well as many invertebrates, possess nervous systems that are capable of detecting noxious stimuli through a process called nociception.

Nociception is an integral part of the nervous system. The mammalian body constantly interprets the outside world through the web of nerve fibers that constitute

the peripheral nervous system. Every single moment, these fibers are detecting, integrating and conveying somatosensory information to the brain, which then interprets the collected "data". These fibers are either specialized to detect a specific type of stimuli or have the capactity to sense multiple types of stimuli. In the skin, there are specialized fibers for detecting heat, cold, harmful chemicals, pressure and vibration (Julius and Basbaum, 2001; Meyer et al., 2006). Nociception is mediated by nociceptors. This special class of neurons is activated by chemical irritants, noxious heat, and potentially harmful pressure, but they are not activated by innocuous stimuli like gentle touch (Burgess and Perl, 1967).

Nociceptors can be divided into different classes based on their eletrophysiological response to certain stimuli (See Figure 1.1). Medium diameter nociceptors with thinly myelinated axons are known as Aδ fibers. These fibers conduct action potentials very rapidly (though not as rapidly as Aβ fibers— a class of sensory neurons which detect innocuous stimuli). Evidence from previous studies suggests that these fibers are responsible for pricking, sharp pain as well as the detection of noxious heat and sensitization following a burn or chemical injury (Campbell et al., 1979; Julius and Basbaum, 2001; Ringkamp et al., 2001; Treede et al., 1998). Unlike the rapid response of Aδ fibers, nociceptors with the smallest diameters conduct action potentials very slowly due to their unmyelinated axons. Called C-fiber nociceptors, these cells are thought to be responsible for burning pain sensations (Julius and Basbaum, 2001; Meyer et al., 2006). Polymodal, they respond to noxious thermal, chemical and mechanical stimuli. With their slow adapting responses to chemical and mechanical stimuli, they have also been

implicated in hyperalgesia, a type of sensitization that occurs following tissue injury (Peng et al., 2003).

The cell bodies of nociceptors reside within the trigeminal and dorsal root ganglia (DRG) that flank the brain stem and spinal cord, respectively. Nociceptors have a pseudo-unipolar morphology with a long axon that bifurcates into two distinct processes (Meyer et al., 2006). The distal fibers travel out of the ganglia to innervate various parts of the body while the proximal processes connect to the central nervous system through synapses in the spinal cord or brainstem. This junction between nociceptors and neurons that ascend to the brain is where critical information transmission occurs in nociception (See Figure 1.2). As such, this junction presents an ideal target for the development of prophylactic pain treatments. Yet, it is only recently that researchers have begun to grasp the complexity of this intricate sensory system.

1-3 Classifying nociceptors based on molecular markers

In addition to their size and electrophysiological properties, nociceptors can also be distinguished based on the expression of molecular markers. The functions of these proteins are varied. They include cell surface proteins, stored peptides, secreted molecules and a number of enzymes (Meyer et al., 2006). While the distribution of markers is not necessarily conserved across species and fluctuates in response to injury, these markers are useful for dividing nociceptors into specific subclasses (Meyer et al., 2006; Zwick et al., 2002). Recent studies have taken

advantage of these markers to categorize these neurons into distinct nociceptive circuits based on anatomy and functionality (Cavanaugh et al., 2009; Zylka et al., 2005). Large diameter neurons with Aβ and Aδ fibers can be identified with the molecular marker neurofilament protein NF200 (Meyer et al., 2006).

In the DRG, small diameter neurons are roughly divided into two classes based on molecular markers: peptidergic and nonpeptidergic (See Figure 1.3) (Julius and Basbaum, 2001; Meyer et al., 2006; Zylka et al., 2005). Peptidergic neurons contain peptide markers like substance P, calcitonin gene-related peptide (CGRP), somatostatin, the capsaicin receptor TRPV1 and TrkA (Fitzgerald, 2005; Meyer et al., 2006). Roughly 40% of small diameter DRG neurons have been classified as peptidergic (Lawson et al., 1996; McCarthy and Lawson, 1989). In addition to these unique markers, peptidergic neurons also project to laminae I and II_{outer} of the dorsal horn in the spinal cord (Hunt and Mantyh, 2001; Ribeiro et al., 2003; Zylka et al., 2005)

Nonpeptidergic neurons are able to bind the plant lectin IB4. They are also distinguished by expression of the ATP gated ion channel $P2X_3$ and prostatic acid phosphatase (PAP, also known as FRAP), an enzyme that has been shown to be antinociceptive in mice (Zylka et al., 2008). It should be noted that there is some overlap between PAP and peptidergic markers like substance P and CGRP. PAP expression, in particular, has been found in both peptidergic and nonpeptidergic neurons. However, nonpeptidergic neurons can ultimately be identified because they specifically innervate lamina II of the dorsal horn (Perry and Lawson, 1998; Zylka et al., 2005).

Though there is some overlap between these two categories of nociceptive neurons, this classification system continues to be useful for defining nociceptors. The segregation of specific proteins in peptidergic versus nonpeptidergic neurons as well as the strict division of their axon terminals in the dorsal horn suggests that there are conserved functions for each class of neurons (Zylka et al., 2005). Continued exploration of these protein markers will ultimately help researchers determine the functional differences between these nociceptor classes and contribute to a better understanding nociception as a whole.

1-4 Purinergic Signaling in Nociception

Purinergic signaling between cells facilitates a number of critical functions which include endocrine secretion, immune response, inflammation, vasodilatation, cell proliferation, cell death and nociception (Burnstock, 2007; Burnstock, 2009). The first mention of the potent extracellular signaling actions of adenine molecules appeared in a seminal paper by Drury and Szent-Gyorgyi in 1929 (Drury and Szent-Györgyi, 1929). The concept of adenine molecule signaling was initially resisted due to the well-established intracellular roles of adenosine triphosphate (ATP)—particularly its role in metabolism. Burnstock later characterized the process further and coined the term “purinergic” signaling in his 1972 review. In the years following his pivotal review, Burnstock further postulated the existence of purinergic receptors and categorized them into two distinct families of nucleoside-activated and nucleotide-activated receptors (Burnstock, 1976; Burnstock, 1978). Around the same time, other studies hinted that ATP could be a pronociceptive compound. But

it took more time for researchers to establish a connection between purinergic receptors and nociception.

1-4.1 *The pronociceptive properties of ATP*

The first hint that ATP was a pronociceptive agent occurred when Keele and Collier washed broken blisters on human skin with an ATP solution in 1964. At the time, the blister base preparation—where blisters are induced then ruptured to "expose" aggravated nerve endings—was one of the best methods available to compare the pain-inducing properties of different compounds. Keele and Collier observed that their subjects recorded higher pain scores when the exposed blisters were treated with ATP. Keele replicated his results with Bleehen a decade later. In the mean time, Collier et al. had established that ATP is pronociceptive in mice. Following these seminal studies, other groups found evidence that the electrophysiological response of nociceptors to ATP was substantially different from nonnociceptive neurons. Despite this progress, the molecular mechanism for ATP stimulated nociception remained unclear.

1-4.2 *P2X and P2Y Receptors*

Following the discovery of purinergic receptors in 1972, the receptors were divided into two groups: one for adenosine activated receptors (P1) and another for ATP/ADP activated receptors (P2). Later on, the P2 receptors were further segregated into a class of ion-gated receptors (P2X) and a class of G protein-

coupled receptors (P2Y). Most of these receptors are conserved across mammalian species and to date twenty-seven purinergic receptors have been identified.

There are fifteen known P2 receptors. Seven are P2X subunits that combine with each other to form trimeric cation channels permeable to Na^+ and Ca^{2+} (Sawynok, 2007). The other eight are P2Y receptors; mebatropic G protein-coupled receptors that activate various signaling pathways. Though both receptor subtypes are activated by ATP and ADP, P2Y receptors can also be activated by pyrimidine and purine nucleosides (Burnstock, 2009). Three P2Y receptors also favor diphosphate substrates like ADP. These differences in substrate specificity and preference suggest P2X and P2Y receptors have different regulatory roles. But how are those roles linked to nociception?

The first major connection between nociception and purinergic signaling was made in 1995 when the $P2X_3$ ionotropic receptor was cloned by two separate groups (Chen et al., 1995; Lewis et al., 1995). The receptor was predominantly localized to small diameter nociceptive neurons in the DRG along with the heteromultimer channel $P2X_{2/3}$. Both groups of investigators demonstrated that the neurons could be activated by ATP *in vitro*. Years later, Jarvis, et al. demonstrated that $P2X_3$ agonists produced an increase in nociceptive behavior *in vivo* (Jarvis and Kowaluk, 2001). Jarvis, et al. also showed that the converse was true: they found that subcutaneous injections of A-317491, a specific $P2X_3$ antagonist, reduced nociceptive behaviors in rats. Knockdown of $P2X_3$ using antisense oligonucleotides also reduced nociceptive behaviors in different models of induced hyperalgesia (Dorn et al., 2004; Honore et al., 2002). Further study has uncovered roles for

these receptors in many different pain modalities including neuropathic, inflammatory, visceral and migraine pain (Wirkner et al., 2007). Yet, these results are complicated by the fact that $P2X_3$ knockout mice show normal sensitivity to noxious stimuli in behavioral tests. However, these mice do display differences in sensitivity once an injury has been induced, suggesting that a form of compensation is occurring before injury. Whether compensation occurs during development or there is increased function of another P2X receptor subtype has yet to be determined.

Once the connection between $P2X_3$ and nociception was firmly established, researchers began to investigate how other P2X receptors affected nociception. To date, three out of seven P2X receptors have been implicated in nociception: $P2X_3$, $P2X_4$ and $P2X_7$. $P2X_4$ receptors were found in dorsal horn neurons as well is in spinal microglia. A recent study showed that knockdown of $P2X_4$ receptors in rats suppressed the development of allodynia following nerve injury (Tsuda et al., 2003). This group also showed that administering antagonists of $P2X_4$ receptors also reduced tactile allodynia *in vivo*. Similar to $P2X_3$, suppressing receptor function with a drug produces different results than genetic ablation of the receptor. $P2X_4$ null mice have normal responses in several nociceptive assays, yet they show different sensitivity following injury (Tsuda et al., 2009). $P2X_7$ receptors are also found on microglia. Antagonists for $P2X_7$ receptors reduce nociception in animal models of neuropathic pain, yet once again the *$P2X_7$-/-* mice demonstrate normal nociceptive behaviors (Honore et al., 2006; Nelson et al., 2006). Furthermore, the *$P2X_7$-/-* animals cannot develop inflammatory or neuropathic pain states (Chessell et al.,

2005). Despite the puzzling situation involving genetic ablation of P2X receptors, these studies nevertheless highlight the importance of P2X receptors in nociception.

Four out of eight known P2Y receptors have possible connections to nociception: $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$. All four receptors were detected in the spinal cord. $P2Y_2$ and $P2Y_4$ were also found in the DRG (Okada et al., 2002). Another study refutes this finding by showing only $P2Y_1$ and $P2Y_2$ are in the DRG (Kobayashi et al., 2006). Despite this minor controversy, the role of P2Y receptors in nociception was investigated in rodents. In order to avoid simultaneous activation of P2X receptors, many researchers took advantage of the fact that P2Y receptors could be activated by pyrimidine as well as purine based substrates. Most groups used either uridine 5'-triphosphate (UTP) or uridine 5'-diphosphate (UDP). Using these substrates, researchers found that P2Y receptors were involved in neuropathic pain; receptor activation relieved the allodynia produced by sciatic nerve injury (Okada et al., 2002). Furthermore, intrathecal injection of UTP and UDP increased the threshold of naïve animals in testing for mechanical nociception (Okada et al., 2002). Pinpointing which receptor is ultimately responsible for these antinociceptive effects as specific antagonists for P2Y receptors have yet to be developed. Since it is known that $P2Y_2$ and $P2Y_4$ have an affinity for UTP while $P2Y_6$ has an affinity for UDP, most authors suggest that the antinociceptive effect must be mediated by one of these three receptors. Also, $P2Y_2$ has recently been connected to thermal nociception and the thermosensor TRPV1 (Malin et al., 2008).

Another angle that investigators are beginning to explore is the interaction between different P2X and P2Y receptors. A recent study by Chen, et al.

demonstrated that $P2X_7$ receptors in microglial satellite cells exert inhibitory control over $P2X_3$ and $P2Y_1$ receptor activity in neurons (Chen et al., 2008). Though these results have yet to be supported by evidence from other groups, the conclusions of Chen, et al. reinforce the importance of P2 receptor signaling in nociception and also highlight the importance of investigating receptor interaction.

1-4.3 *The antinociceptive properties of adenosine*

While ATP stimulates nociception, adenosine produces the opposite effect. The first antinociceptive effect of adenosine was observed by Vapaatalo, et al. in 1975 when they systemically treated rats with the adenosine analog phenyliso-propyl-adenosine. The effect was confirmed by other studies in which drugs were administered systemically or delivered directly to the nervous system (Holmgren et al., 1983; Yarbrough and McGuffin-Clineschmidt, 1981). A key advancement in the field was made when two separate groups determined that the antinociceptive effect of adenosine was occurring through a spinal site (Holmgren et al., 1986; Post, 1984). Given that two subtypes of adenosine activated purinergic receptors were localized to the spinal cord (Choca et al., 1987; Geiger et al., 1984; Goodman and Synder, 1982), the investigators hypothesized that the antinociceptive effects were mediated by adenosine receptors.

1-4.4 *Adenosine Receptors*

When Burnstock postulated the existence of adenosine receptors in 1976, the concept of adenosine as a signaling molecule was hardly new. Since the initial

discovery by Drury and Szent-Gyorgyi that showed adenine-containing compounds had profound effects on the mammalian heart, numerous other studies have also shown that adenosine as well as adenosine analogs can affect many different bodily functions (Barsoum and Gaddum, 1935; Cobbin et al., 1974; Drury, 1936; Drury and Szent-Györgyi, 1929; Stafford, 1966). Burnstock initially named the receptors "P1" receptors in line with his naming of the "P2" receptors. This nomenclature was later abandoned in favor of terminology that described the endogenous agonist of the receptors (Fredholm et al., 2001). Currently, there are four known adenosine receptors: A_1, A_{2A}, A_{2B} and A_3. All adenosine receptors are G protein-coupled, seven transmembrane domain receptors.

Of the four known adenosine receptors, the A_1 adenosine receptor (A_1R) is the most firmly connected to nociception. A_1R in the nervous system is expressed in DRG, trigeminal ganglion neurons and throughout the spinal cord (Carruthers et al., 2001; Reppert, et al.,1991; Schulte et al., 2003). *In vitro* studies have shown that activation of A_1R decreases the release of the inflammatory substances CGRP and substance P (Sjolund et al., 1997). Corresponding *in vivo* studies demonstrated that adenosine and adenosine analogs lead to antinociceptive effects through the activation of A_1R (Aley et al., 1995; Aumeerally et al., 2004; Karlsten et al., 1992; XJ and Sawynok, 2001). Spinal and peripheral delivery of A_1R agonists lead to similar antinociceptive effects (Dickenson et al., 2000; Sawynok, 1998). Investigators have also successfully activated A_1R by manipulating the levels of endogenous adenosine; specifically, inhibitors of adenosine kinase and adenosine deaminase were antinociceptive in models of inflammatory and neuropathic pain (Jarvis et al.,

2002a; Kowaluk and Jarvis, 2000; McGaraughty et al., 2001; McGaraughty et al., 2005). Generation of A_1R knockout mice further confirmed the importance of this receptor in nociception. *$A_1R^{-/-}$* mice have enhanced thermal sensitivity in the tail flick assay and develop enhanced thermal sensitivity following inflammation or nerve injury (Johansson et al., 2001; Wu et al., 2005). Though these animals have normal responses to mechanical stimuli at baseline and following injury, they do not respond as well to conventional pain treatments like morphine (Wu et al., 2005). Ultimately, these data suggest a key role for A_1R in regulating thermal nociception as well as participating in the mechanism of morphine analgesia.

Unlike A_1R, A_{2A}, A_{2B} and A_3 receptors are considered to be pronociceptive. $A_{2A}R$ is expressed in DRG neurons, glial cells and in the spinal cord (Bura et al., 2008; Cunha et al., 2006; Haskó et al., 2005; Hussey et al., 2007; Kaelin-Lang et al., 1998). Studies have consistently shown that activation of $A_{2A}R$ in the peripheral nervous system leads to increased nociceptive behaviors in animals, whereas suppression of $A_{2A}R$ activity has led to a reduction in nociceptive behaviors (Doak and Sawynok, 1995; Khasar et al., 1995; Taiwo and Levine, 1990). Yet, the effect of $A_{2A}R$ activation in the central nervous system remains unclear. Both $A_{2A}R$ agonists *and* antagonists lead to antinociception when injected into the spinal cord (Godfrey et al., 2006; Hussey et al., 2007; Loram et al., 2009; Regaya et al., 2004; Yoon et al., 2005). The $A_{2A}R$ knockout mice show reduced nociceptive responses to thermal tests, decreased thermal hyperalgesia and mechanical allodynia as well a reduced response to formalin and opioids (Bura et al., 2008; Godfrey et al., 2006; Ledent et al., 1997). As such, the overall effect of $A_{2A}R$ on nociception is unclear. $A_{2B}Rs$ and

A_3Rs are located in mast cells in the peripheral nervous system. Activation of both these receptor subtypes leads to the release of inflammatory substances like histamine and serotonin. The knockout mice for both receptors show largely normal responses in nociceptive tests except for one report of A_3R knockout mice demonstrating reduced thermal nociception in the hot plate test. Ultimately, further study is needed of all these receptor subtypes to pinpoint their specific roles in nociception.

1-4.5 *Sources of Endogenous Adenosine*

The fact that adenosine receptor activity is so critical to nociceptive signaling in both the peripheral and central nervous systems implies that there are endogenous mechanisms for generating adenosine. Though the precise amount of adenosine in the spinal cord is unclear, basal adenosine levels in other locations of the CNS are enough to activate high affinity adenosine receptors such as A_1 or A_{2A} (Dunwiddie and Masino, 2001). This activation implies that adenosine signaling is a constitutive process in the CNS. As such, injury or other pathologies likely stimulate the generation of additional adenosine. Production of this adenosine occurs primarily through two different mechanisms in the spinal cord: intracellular release from neurons or the dephosphorylation of released adenosine nucleotides (See Figure 1.4).

Intracellular ATP is abundant and is a key source of adenosine (Sawynok and Liu, 2003). The enzyme ATPase breaks ATP down into AMP. Then an enzyme called intracellular 5'-nucleotidase ((cN)-I) hydrolyzes AMP into adenosine.

Adenosine can also be generated from the metabolism of cAMP our from the compound S-adenosyl-homocysteine (SAH) (Latini and Pedata, 2001). Cells can then release adenosine into the extracellular space through equilibrative nucleoside transporters or generate inosine for other signaling purposes. Adenosine release can be stimulated in response to compounds associated with injury and inflammation, including capsaicin and substance P (Cahill et al., 1997; Sweeney et al., 1989). Release can also occur in response to analgesic drugs such as morphine (Sandner-Kiesling et al., 2001; Sweeney et al., 1987a; Sweeney et al., 1987b). NMDA, potassium and serotonin have been shown to trigger adenosine release as well (Conway et al., 1997; Sweeney et al., 1987a; Sweeney et al., 1989).

The other method of generating adenosine in the extracellular space is enzyme-mediated dephosphorylation of adenine-containing nucleotides released from synaptic vesicles. ATP is a neurotransmitter and neuromodulator that can act specifically on P2X and P2Y receptors (Dunwiddie and Masino, 2001). In order to minimize action of these receptors, ATP is quickly dephosphorylated by enzymes called ectonucleotidases. These enzymes form a “purinergic cascade” in which ATP is ultimately broken down into adenosine. From that point, adenosine can either act on adenosine receptors, be taken back up into cells or be converted into inosine. Adenosine that enters cells can either be phosphorylated into AMP by adenosine kinase or deaminated into inosine by adenosine deaminase. Blocking the activity of these intracellular enzymes has been shown to increase adenosine release, leading to antinociceptive effects *in vivo* (Latini and Pedata, 2001). But more importantly,

the manipulation of extracellular ectonucleotidases leads to profound effects on nociception.

1-5 Ectonucleotidases in Nociception

Ectonuleotidases are a class of nucleotide-metabolizing enzymes that are found on the plasma membrane of cells. They are distributed throughout the body and perform multiple regulatory functions in various tissues (for a comprehensive review, see (Yegutkin, 2008)). As mentioned in section 1-4-5, these enzymes are largely responsible for dephosphorylation of ATP to adenosine. As such, they have a key role in the regulation of purinergic receptor activity. The nervous system contains members from all the ectonucleotidase families: ectonucleoside triphosphate diphosphohydrolases (ENTPD), ectonucleotide pyrophosphatase/phosphodiesterases (ENPP), alkaline phosphatases (AP), acid phosphatases and 5'-nucleotidases (Zimmermann, 2006). Of these various enzymes, two have been firmly connected to nociception: an acid phosphatase called prostatic acid phosphatase (PAP) and a 5'nucleotidase called ecto-5'-nucleotidase (NT5E).

1-5.1 *Prostatic Acid Phosphatase*

Early histochemical studies determined that there was an enzyme located in lamina II of the spinal cord and specific neurons of the DRG that could dephosphorylate thiamine monophosphate (TMP). Initially named fluoride resistant acid phosphatase (FRAP) or TMPase, numerous groups studied the enzyme during

the 1970s and the1980s, but were unable to determine what gene encoded the enzyme (Dodd et al., 1983; Sanyal and Rustioni, 1974; Silverman and Kruger, 1988a). Many investigations noted that TMPase was localized to small diameter nonpeptidergic DRG neurons, suggesting that there was a connection between TMPase and nociception. A few groups even showed that TMPase expression was reduced or eliminated when peripheral nerves were damaged (Shields et al., 2003a; Tenser, 1985; Tenser et al., 1991). Upon discovery of another, easier to use, marker for nonpeptidergic neurons called isolectin B4(IB4), many investigators dropped their pursuit of this elusive enzyme (Silverman and Kruger, 1988b, 1990).

Dodd, et al. made the most ambitious attempt to identify the gene when they purified TMPase from rat DRGs. They found that the protein could be inhibited by the nonselective acid phosphatase inhibitor, L(+)-tartrate and the protein was similar in size to a secretory human protein found in the prostate—prostatic acid phosphatase(PAP). At the time, however, a decent PAP antibody was not available and the one used by Dodd, et al. failed to stain the small diameter nociceptive neurons where TMPase was located. As such, they were unable to provide conclusive evidence that TMPase was PAP.

Decades later, our lab revisited this mystery. With in situ hybridization we showed that small diameter nociceptive neurons specifically express a transmembrane isoform of the PAP enzyme; thereby explaining why the protein Dodd et al. obtained was larger than the secretory form. TMP lead histochemistry and immunohistochemistry confirmed that PAP was expressed in the DRG and also showed that the enzyme was expressed in lamina II of the dorsal horn (Zylka et al.,

2008). Additional characterization using molecular markers for peptidergic and nonpeptidergic neurons showed that PAP was primarily expressed in nonpeptidergic neurons, just like TMPase. But the most convincing evidence that TMPase was PAP arose when these procedures were repeated in *PAP-/-* mice—the staining in the DRG and dorsal horn was completely eliminated (See Figure 1.5).

In addition to identifying this mysterious CNS enzyme, our lab also discovered that secreted PAP protein has antinociceptive properties. When injected intrathecally (i.t.) in mice, PAP protein profoundly increased pawwithdrawal latency to noxious thermal stimuli in naïve mice (Sowa et al., 2009; Zylka et al., 2008). This effect lasted for up to three days and was dose-dependent. When compared to the traditional opioid analgesic morphine, PAP's analgesic effect lasted eight times longer with no observable side effects. PAP was also antinoceptive in two models of chronic pain: the complete Freund's adjuvant (CFA) model of inflammatory pain and the spared nerve injury (SNI) model of neuropathic pain. Additional behavioral tests in $PAP^{-/-}$ mice revealed that the animals had enhanced nociceptive responses following injury or inflammation. This phenotype was rescued when $PAP^{-/-}$ mice were injected i.t. with PAP protein.

PAP's ability to function as an ectonucleotidase led us to suspect that the enzyme could be connected to the antinoceptive activity of A_1 adenosine receptors. The selective A_1R agonist N^6-cyclopentyladenosine (CPA) produced similar, though not as long-lasting, antinociceptive effects in our behavioral tests (Zylka et al., 2008). Using lead histochemistry and HPLC we demonstrated that PAP was capable of dephosphorylating AMP into adenosine *in vitro* (See Figure 1.6). Behavioral testing

using $A_1R^{-/-}$ mice further showed that PAP injections were ineffective in the knockout animals. Whereas the wildtype mice displayed decreased sensitivity, the $A_1R^{-/-}$ mice remained highly sensitive to nociceptive stimuli throughout the testing period. Similar results were obtained when the $A_1R^{-/-}$ animals were tested in the CFA and SNI models. These tests definitively confirmed that PAP's antinociception was being mediated by A_1R.

1-5.2 *Ecto-5'-nucleodtidase*

A key observation that was made during our study of PAP was that there was residual AMP histochemical staining in spinal cords and DRGs from *Pap*$^{-/-}$ animals. This suggested that another AMP hydrolyzing enzyme was present in the CNS (See Figure 1.6). We determined that PAP was colocalized with another ectonucleotidase called ecto-5'-nucleotidase (NT5E, also known as CD73) (See Figure 1.7). Though other 5'-nucleotidases exist in humans, NT5E is the only enzyme found on the cell surface. Anchored to the membrane by a GPI anchor, this enzyme selectively hydrolyzes 5'-monophosphates and shows little affinity for hydrolyzing di- or triphosphates (Zimmermann, 1992). This hydrolytic activity can either be enhanced by binding the divalent cations Mg^{2+} or Zn^{2+} or reduced by the cations Pb^{2+} or Hg^{2+} (Fini et al., 1990; Ong et al., 1990). NT5E also functions best in a pH range of 7-8.

Studies by Zimmerman have shown that 5'-AMP is NT5E's preferred substrate (1992). As such, NT5E is proposed to be a key regulator in purinergic signaling in many systems. NT5E has been connected to various physiological processes including, epithelial ion transport, permeability of intestinal and vascular

tissues, hypoxia, ischemic preconditioning in the heart and kidneys as well as inflammation (Colgan et al., 2006). NT5E has also been connected to lung function, renal function and the regulation of immune responses. These studies reflect the near ubiquitous expression of NT5E in many tissues. Through biochemical and histochemical assays, NT5E expression has been detected in the liver, heart, blood vessels, lung, colon, kidney, the brain and the spinal cord (Moriwaki et al., 1999; Yegutkin, 2008; Zimmermann, 1992, 1996). Another recent study in our lab has confirmed the expression of NT5E in the dorsal horn of the spinal cord as well as the DRG (Sowa et al., 2010). Despite the detection of NT5E in the nervous system, a functional role for NT5E in these tissues has yet to be determined.

A huge barrier to functional studies of NT5E has been the lack of purified, soluble, mammalian NT5E protein (Colgan et al., 2006). Most studies of NT5E until recently have relied on studying the enzyme through cell extracts, lysed tissue and tracking the degradation of 5'-AMP to determine the presence and/or activity level of mNT5E. Unfortunately by using these approaches, the possibility remains that other enzymes which degrade 5'-AMP are also dephosphorylating the substrate. To address this caveat, many labs have used a 5'nucleotidase protein purified from rattlesnake (*Crotalus atrox)*, venom in their studies. Like NT5E, snake venom 5'-nucleotidase favors monophosphate substrates, especially AMP, and can bind divalent cations (Iwanaga and Suzuki, 1979). Injections of *C. atrox* 5'-nucleotidase rescued phenotypes in *Nt5e*$^{-/-}$ mice in three independent studies (Eckle et al., 2007b; Hart et al., 2008; Thompson et al., 2004). Nevertheless, all three studies focused on the vascular role of NT5E, not the function of NT5E in the nervous system. The

experiments detailed in this dissertation describe a functional role for NT5E in nociception.

1-6 Research Rationale

To date, there have been many studies that have established the presence of NT5E in the nervous system, yet a physiological role for this enzyme in nervous tissues has not been established. A recently published study by our lab has determined that NT5E is localized to small diameter nociceptors in the DRG (Sowa et al., 2010). This study additionally observed that *Nt5e*$^{-/-}$ mice display more sensitivity to nociceptive stimuli— similar to *Pap*$^{-/-}$ mice. Injection of AMP into these knockout animals also produced a decreased antinociceptive response compared to wildtype animals. This antinociceptive effect did not occur when *A*$_1$*R*$^{-/-}$ animals were injected with AMP, suggesting that NT5E has a role in processing adenosine for A_1R receptors. Though this study established a correlation between NT5E and nociception, the exact role of NT5E in nociception could not be confirmed without soluble NT5E protein.

A previous attempt to generate a recombinant version of secreted rat NT5E was attempted by Servos and colleagues in 1998. Though they successfully generated and purified a catalytically active protein, they did not detect the protein in the culture medium. Instead, most of the protein was stuck in the cell pellet used for the purification, suggesting that Servos and colleagues failed to completely remove the GPI anchor. Even though this method produced active protein, the difficulty in

getting the protein to secrete would likely prevent investigators from collecting sufficient amounts of protein to use for *in vivo* testing.

The following dissertation details the generation of a secreted, recombinant mouse NT5E protein (mNT5E) and the characterization of that protein using both *in vitro* and *in vivo* methods. Our reasons for generating this protein were three-fold: 1) to address toxicity concerns associated with introducing an enzyme derived from snake venom to the nervous system; 2) to design a vector for a secreted version of NT5E that can be easily produced in baculovirus and other organisms used for protein purification; 3) to definitively establish NT5E's participation in nociception. Given the role of the ectonucleotidase PAP in nociception, we hypothesized that NT5E would also produce long-lasting antinociceptive effects *in vivo*. Furthermore we predicted that these antinociceptive effects are dependent on A_1R. This research could ultimately lead to the development of alternative therapies for intractable pain as well as provide a key tool for the study of NT5E in other physiological processes.

1-7 Figures

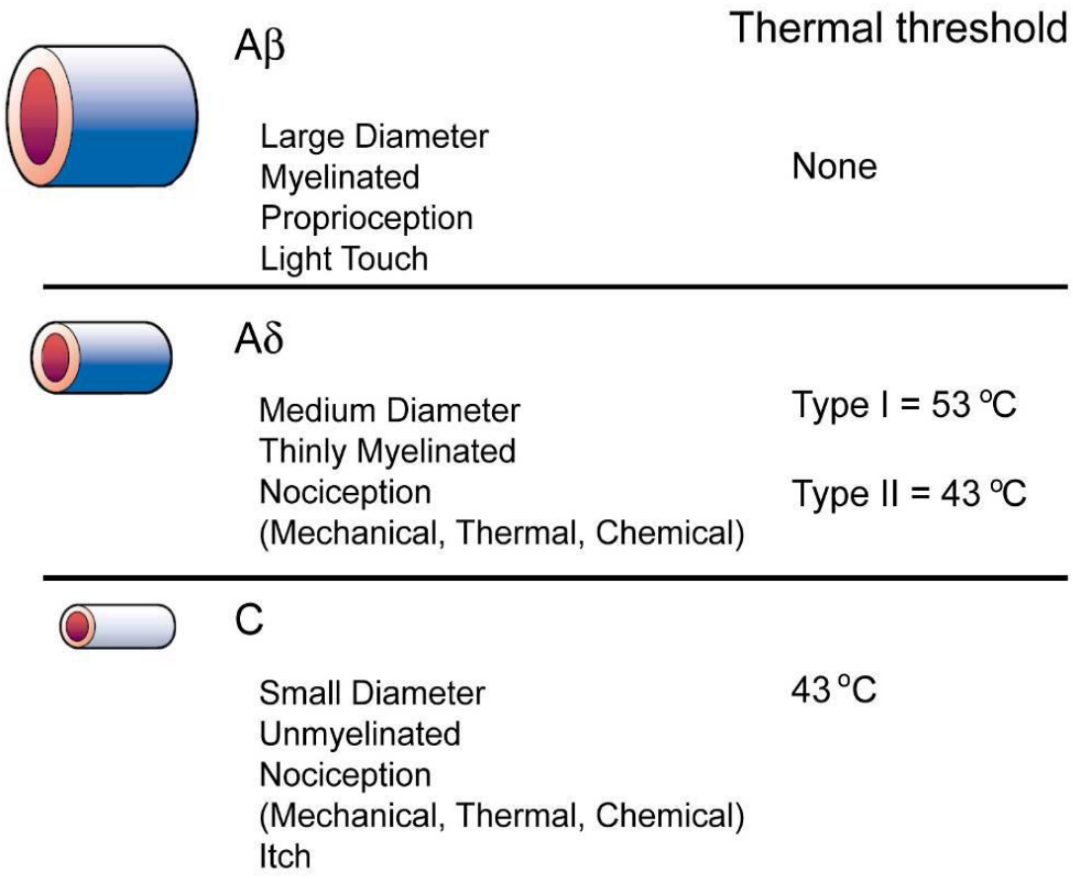

Figure 1.1 Classification of primary afferent nerves. The nerves that make up the peripheral nervous system have different properties and detect various types of stimuli. (Figure modified from Julius and Basbaum, 2001). (Back to text)

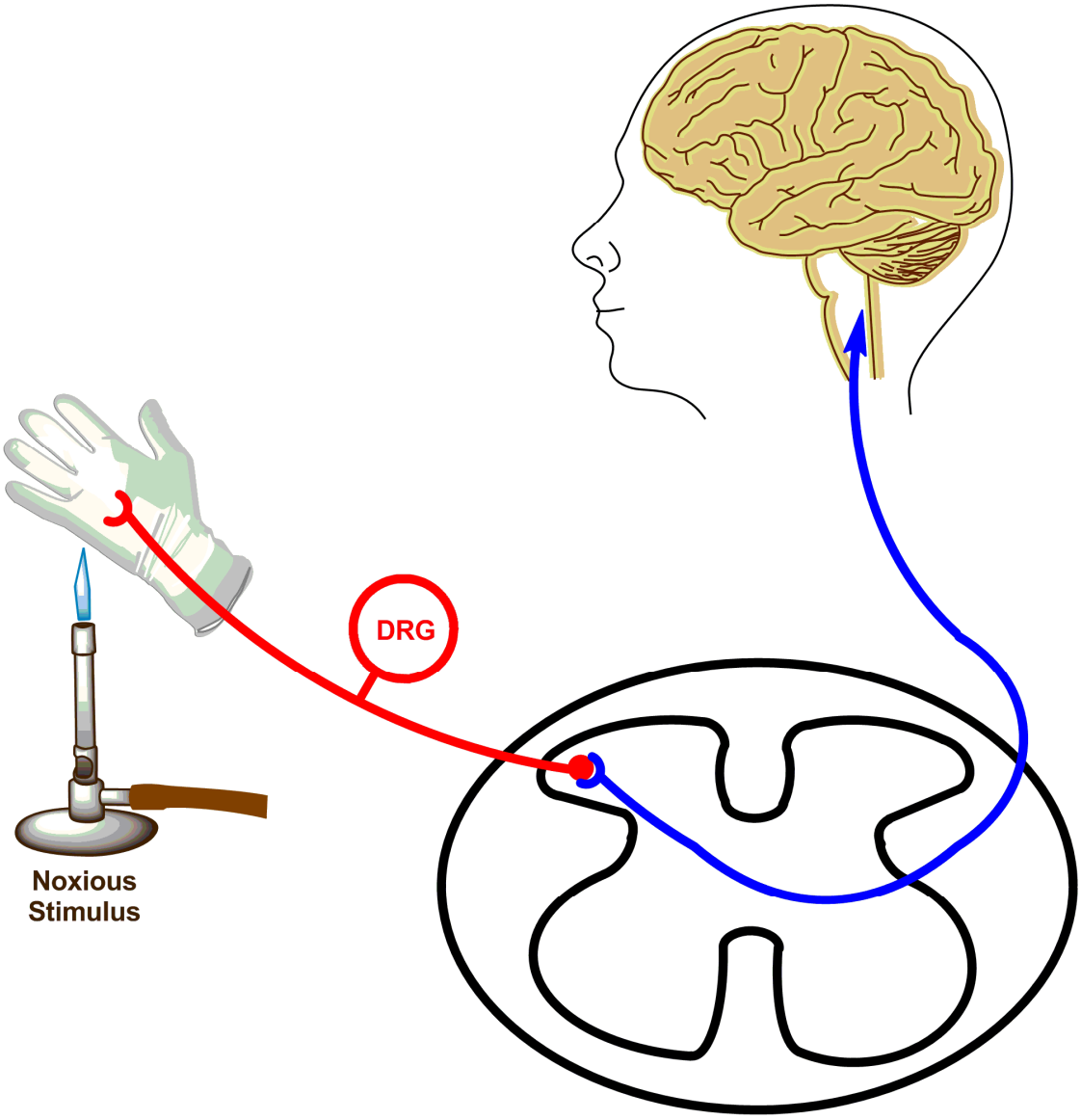

Figure 1.2 Model of peripheral and central nociceptive pathways. Harmful stimuli are detected by nociceptors (red line) in the peripheral nervous system and carry that information to projection neurons that ascend to the brain through the spinal cord (blue line). The critical synapses in this process are found in the dorsal horn. (Back to text).

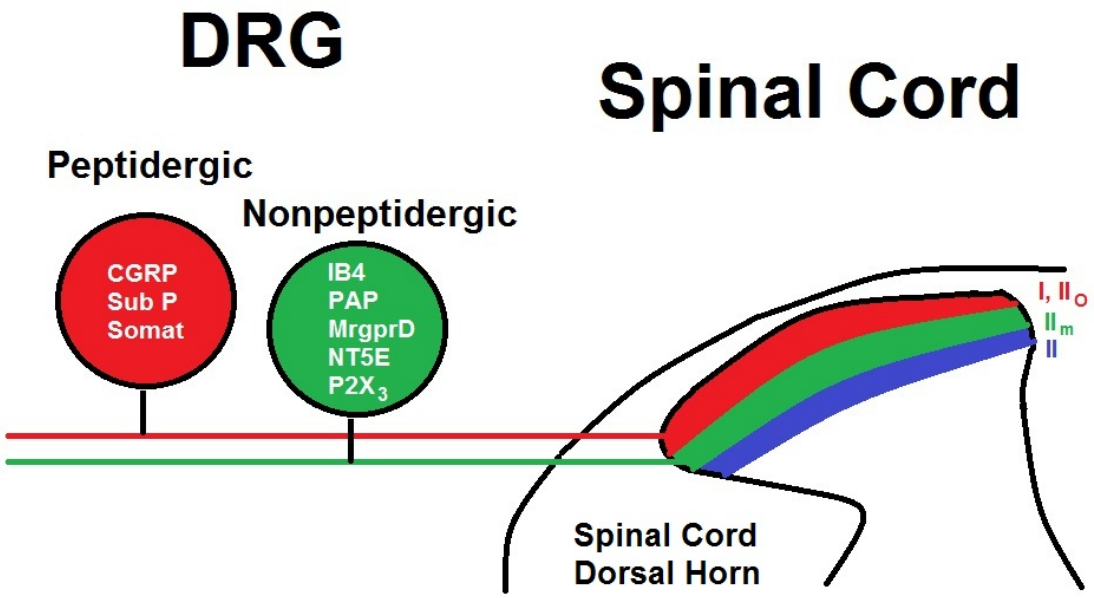

Figure 1.3 Nociceptive neurons are categorized based on molecular markers. Peptidergic neurons (red) express CGRP, substance P (Sub P) and somatostation (somat) and they synapse with neurons in laminae I and II in the dorsal horn of the spinal cord. Nonpeptidergic neurons (green) stain for IB4 and express PAP, MrgprD, $P2X_3$ receptors and NT5E. These neurons project to lamina II. Lamina II middle is a proposed subsection of lamina II inner since the majority of nonpeptidergic neurons terminate in this location . (Figure is modified from Zylka, 2005). (Back to text)

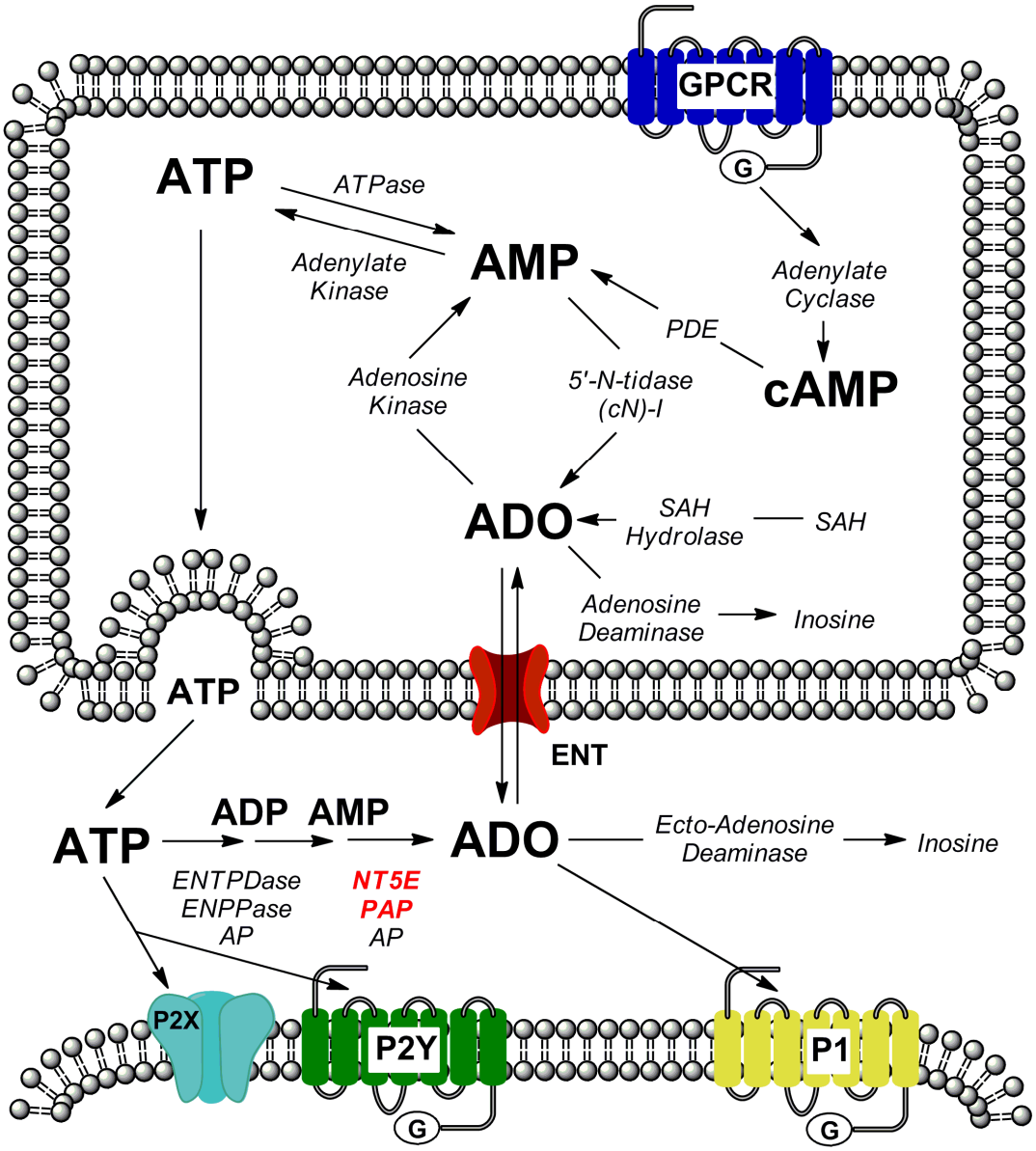

Figure 1.4 Intracellular and extracellular adenosine metabolism. Inside cells, adenosine (ADO) can be generated from three sources: ATP, cAMP or SAH. Outside the cell, adenosine either comes from release through equilibrative nucleoside transporters (ENT) or catabolism of ATP. See text for details. PDE= Phosphodiesterase. (Figure modified from Sawynok and Liu, 2003). (Back to text)

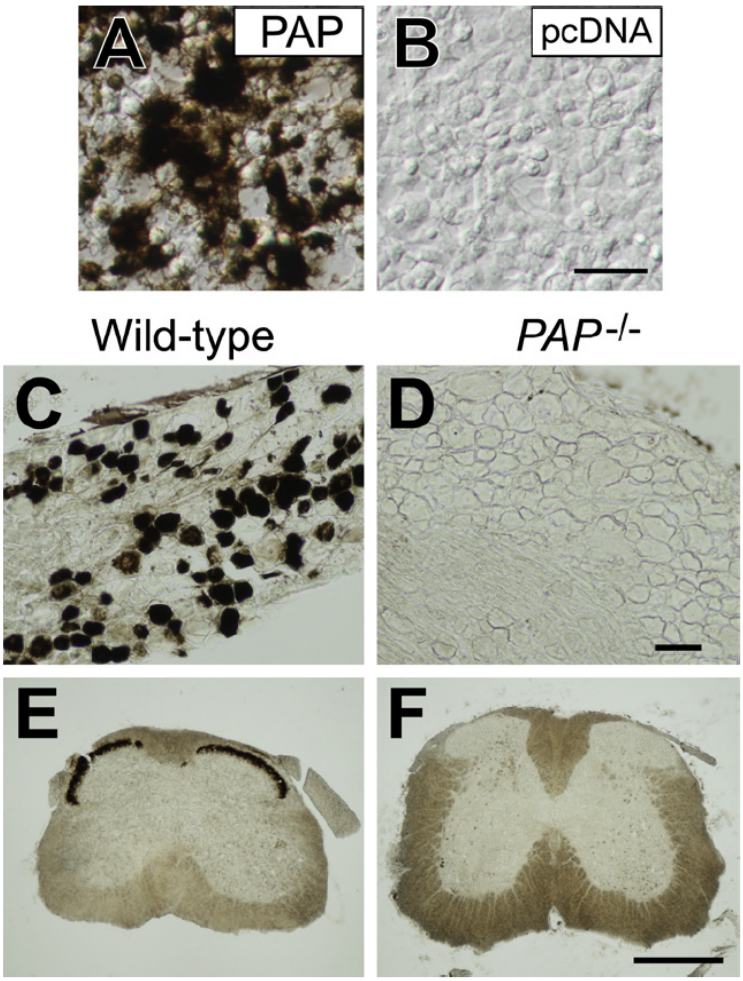

Figure 1.5 PAP Dephosphorlates TMP in nociceptive circuits. (A) HEK 293 cells were transfect with a mouse TM-PAP expression construct or (B) an empty pcDNA3.1 vector then stained with TMP Histochemistry. Lumbar DRG (C and D) and spinal cord (E and F) from wild-type and *Pap-/-* mice were stained using TMP histochemistry. 6 mM TMP was used as a substrate and the buffer was at pH 5.6 for all panels. Scale bar, 50 µm in A and B, 500 µm in C and D. Figure from Zylka, et al., 2008. (Back to text)

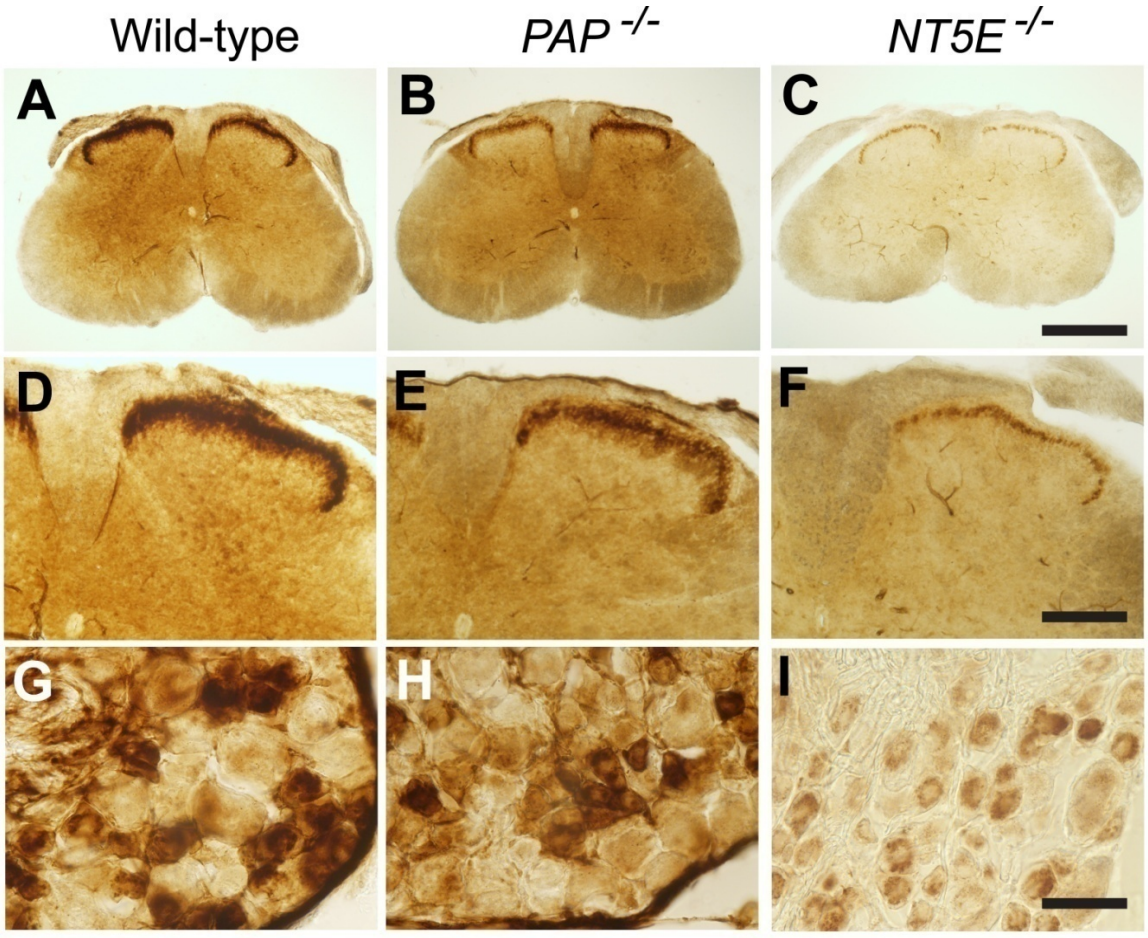

Figure 1.6 PAP and NT5E dephosphorylate AMP in nociceptive circuits Spinal cord (A-F) and DRG tissue (G-I) from wild-type, *Pap*$^{-/-}$ and *Nt5e*$^{-/-}$ mice were stained using AMP histochemistry. 6 mM AMP and a buffer at pH 7.0 were used for all panels. Scale bar, 500 µm for A-C, 200 µm for D-F, and 50 µm for G-I. Figure modified from Zylka, et al., 2008 and Sowa, et al., 2010. (Back to text).

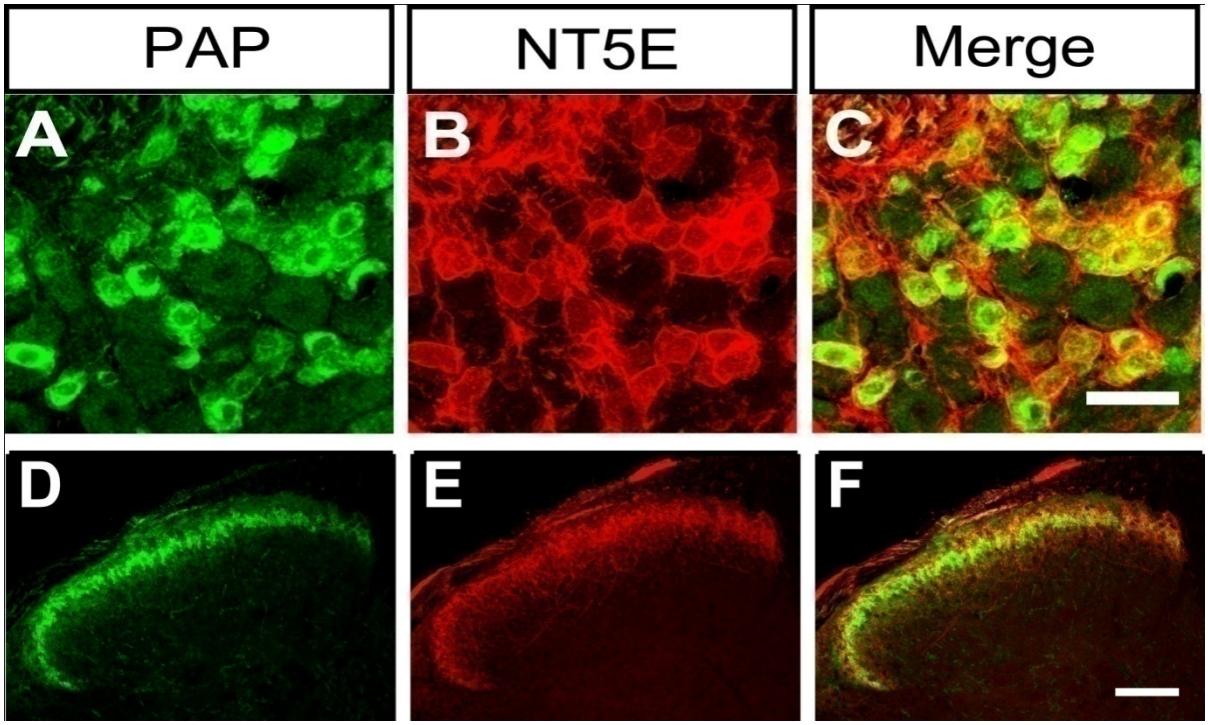

Figure 1.7 PAP and NT5E are colocalized in the Dorsal Horn and DRGs. DRG (A-C) and spinal cord (D-F) tissue from adult mice was stained using anti-PAP and anti-NT5E antibodies. Figure was modified from Sowa, et al., 2010. (Back to text)

CHAPTER TWO

RESEARCH DESIGN AND METHODS

2-1 Molecular Biology of mNT5E recombinant protein

The GST-mNT5E-$(His)_6$ baculovirus expression plasmid was generated by PCR amplification of nt 131-1696 (from GenBank accession # NM_011851.3) using Phusion polymerase and a full-length expression construct of mNT5E as template. Primer sequences contained EcoRI sites (underlined) to facilitate cloning into pAcSecG2T (BD Biosciences). N-terminal primer: 5'-cgcgaattcattgggagctcacgatcctgcacaca. C-terminal $(His)_6$ tag primer: 5'-gcggaattcttaatgatgatgatgatggaacttgatccgcccttcaacg. These primers produce a product that contains the catalytic domain of mNT5E fused to the $(His)_6$ epitope tag but that lacks the signal peptide and GPI anchor sequence (located at Ser523) of mNT5E. The final plasmid was sequence verified. There is only one thrombin cleavage site in the coding region of this plasmid, located between the GST tag and mNT5E coding sequence (See Figure 2.1). .

2-2 Purfication of the mNT5E recombinant protein

The GST-mNT5E-$(His)_6$ plasmid was used to generate recombinant mNT5E protein using the BD BaculoGold Expression System (BD Biosciences). Briefly, we infected Hi5 insect cells with high-titer recombinant baculovirus, incubated the cells

for 48 hours at 27℃ and then removed the cells from the supernatant by centrifugation. The supernatant containing secreted GST-mNT5E-$(His)_6$ was filtered (0.45 μm pore size, Millipore) and concentrated using a Millipore cartridge with a 10k retention cutoff. During concentration, the buffer was exchanged for PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.4). The concentrated supernatant was loaded onto a 5 mL GSTrap FF column (GE Healthcare) using a peristaltic pump at 4℃. Loading was performed overnight at a slow flow rate (0.4 mL/min. for 14-16 hours) to optimize binding of the GST-tagged protein. The column was then washed with 50 mL PBS. Purified thrombin (GE Healthcare, Cat. # 27-0846-01) was added to 2 mL of PBS (250 U thrombin/L of expression culture) and loaded onto the GSTrap column using a syringe. On-column cleavage was allowed to take place for 16 hours at room temperature. The pre-loaded GSTrap column was then attached to an ÄKTA Explorer chromatography system with UV monitoring. Cleaved mNT5E and thrombin were eluted with PBS (the GST tag remained bound to the column). Fractions were monitored with SDS-PAGE to estimate purity, mNT5E concentration and cleavage efficiency (which was ~80%). The cleaved mNT5E was separated from thrombin using a Superdex75 10/300 GL column attached to the ÄKTA Explorer system. Proteins were eluted in PBS at a flow rate of 0.5 mL/min. A maximum of 500 μL was injected per run. Fractions containing cleaved mNT5E were pooled, concentrated and then dialyzed against 0.9% saline. Protein purity was confirmed by SDS-PAGE, staining for total protein with GelCode Blue (Pierce/Thermo Scientific, Cat. # 24590) and western blotting with anti-NT5E antibody (R&D Systems, AF4488). Amersham full-range rainbow molecular weight

markers (GE Healthcare) were used for SDS-PAGE and western blots. Although mNT5E could bind to a nickel chelate column via the $(His)_6$ epitope tag and be eluted with imidazole, we found this additional affinity purification step was unnecessary. Recombinant mNT5E was kept at 4℃ for short- term (1-2 months) use and at -80℃ for long-term storage.

2-3 Enzymatic Assays of mNT5E

Enzymatic reactions (50 μL final) were carried out with recombinant mNT5E at 37℃ for 3 minutes in 100 mM HEPES, pH 7.0, 4 mM $MgCl_2$ with adenosine 5'-monophosphate (AMP disodium salt, Fluka, 01930) 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 incubated 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). Unit (U) definition: 1 U hydrolyzes 1 nmol of AMP per minute at 37℃ at pH 7.0. α,β-me-ADP was purchased from Sigma (M3763).

2-4 Behavioral Testing of mNT5E

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 mice, 2-4 months old, were purchased from Jackson Laboratories. $A_1R^{-/-}$ mice were backcrossed to C57BL/6J mice for 12 generations

(Hua et al., 2007; Johansson et al., 2001). Male mice were used for all behavioral studies and were acclimated to the testing room, equipment and experimenter for at least three days before testing. To further reduce variability in behavioral studies, mice were almost exclusively tested when in the resting or light sleep behavioral state (Callahan et al., 2008). The experimenter was blind to genotype 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 time point to determine paw withdrawal latency (See Figure 2.2 A,B). 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). The force values obtained with this apparatus are higher than the force values obtained using calibrated von Frey filaments (Inoue et al., 2004) (See Figure 2.2 C,D). Three measurements were taken from each paw 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. We performed spared nerve injury surgeries as described by Shields and colleagues (Shields et al., 2003b). mNT5E protein was diluted in 0.9% saline for intrathecal injection (5 μL / mouse) using the direct lumbar puncture method (Fairbanks, 2003) (See Figure 2.2 E,F).

None of the mNT5E-injected mice displayed reduced mobility or paralysis following injection, as assessed by visually observing motor activity following injections.

2-5 Figures

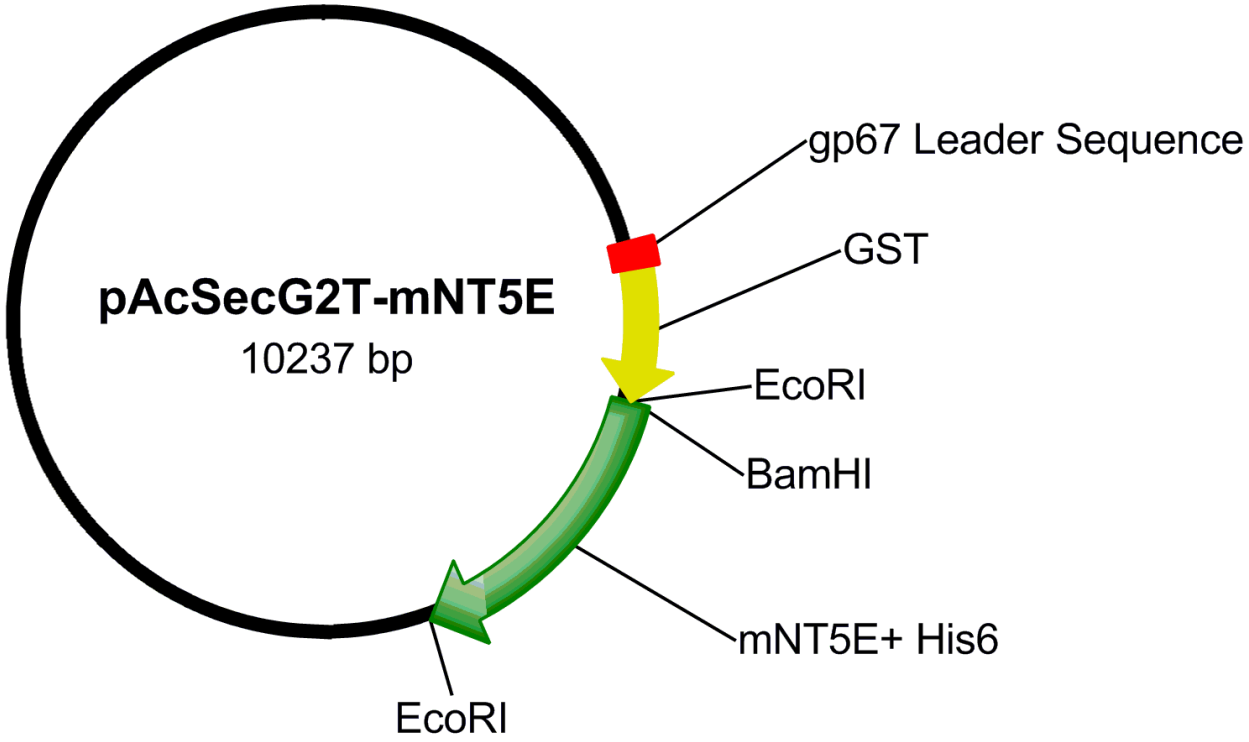

Figure 2.1 Vector map of the mNT5E plasmid. The catalytic domain of mNT5E, which lacks the signal peptide and GPI anchor sequence (located at Ser523) of mNT5E, was fused to a $(His)_6$ epitope tag and inserted into the baculovirus vector pAcSecG2T using EcoRI restriction sites. (Back to text)

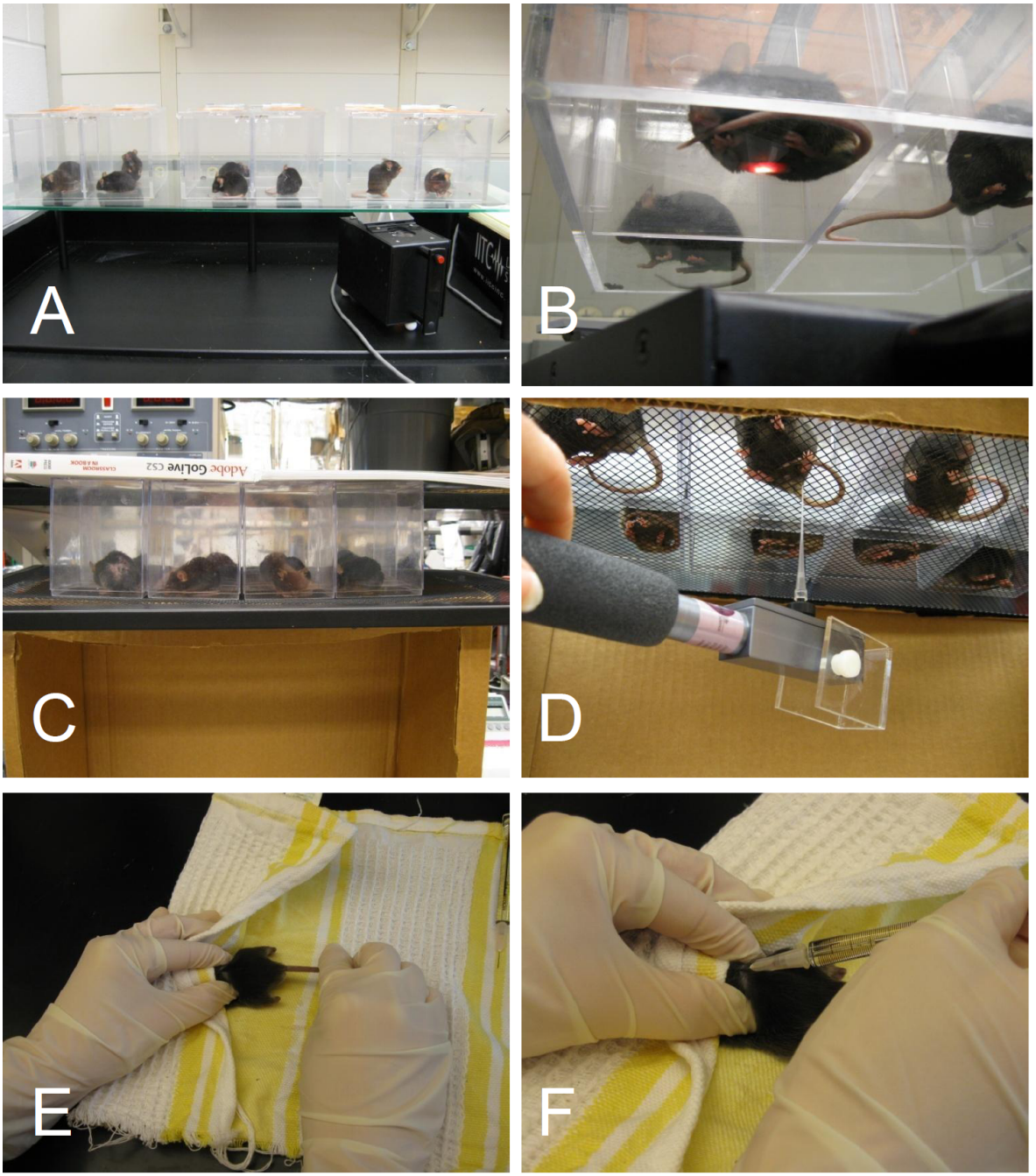

Figure 2.2 Behavioral techniques for testing nociception. To test thermal sensitivity, animals were placed in a Hargreaves apparatus (A) and their paws were heated with a radiant heat source (B). To test mechanical sensitivity, animals were

placed in individual plastic boxes on a raised wire mesh screen (C) and their paws were stimulated through the wire with semi-flexible tips on an Electronic Von Frey device (D). For many of our experiments, animals were injected intrathecally. To accomplish this, an unanestitized mouse would be restrained by the hips with the fron half of the animal covered by a towel (E). Then a 30 guage ½ inch needle attached to a Hamilton syringe was slipped between the lumbar vertebrate (F). The drug was delivered when the animal's tail "flicked", which indicates successful puncture of the dura. (Back to text)

CHAPTER THREE

RESULTS

3-1 Purification of secreted mouse NT5E using the baculovirus expression system

Mature NT5E is anchored to the membrane via a GPI linkage on Ser523 (Ogata et al., 1990). In an effort to generate and purify a secretory (non-membrane anchored) version of NT5E, Servos and colleagues generated a baculovirus expression construct that contained rat NT5E fused to the signal peptide of gp67 and that reportedly lacked the GPI-anchor at Ser523 (Jörg Servos et al., 1998). While Servos and colleagues successfully generated and purified catalytically active rat NT5E, they did not detect NT5E in the tissue culture medium as would be expected for a secreted protein. Instead, NT5E was only present in cell lysates. Since we were interested in purifying a secretory version of mouse NT5E (mNT5E) from the culture medium, we carefully re-examined the cloning strategy used by Servos and colleagues. We noticed that their C-terminal PCR primer actually included Ser523 but excluded the near adjacent Ser526. Using GPI prediction software (Eisenhaber et al., 1999), we confirmed that Ser523 (but not Ser526) was the most likely GPI anchor site.

Since inclusion of this GPI anchor sequence could explain why Servos and colleagues were unable to detect NT5E in the culture medium, we generated a new

expression construct that was virtually identical to the one used by Servos and colleagues except that our construct contained mouse NT5E (Trp29-Phe522; not rat NT5E) that was truncated just before Ser523 (See Figure 3.1A). Our construct also contained the gp67 signal peptide, Glutathione S-transferase (GST), a thrombin cleavage site to permit removal of GST and a C-terminal hexahistidine $(His)_6$ tag.

Two days after infecting Hi5 insect cells with recombinant baculovirus we detected GST-mNT5E protein in the tissue culture supernatant at approximately 10 mg/L. This observation suggested that exclusion of Ser523 was important for producing a secreted version of NT5E. Additionally, based on SDS-PAGE and western blotting, the GST-mNT5E found in the medium was largely intact whereas crude cell lysates contained truncated as well as intact versions of mNT5E (data not shown). We next purified mNT5E from the culture supernatant in two steps (see Methods). We reasoned that GST, a protein that binds glutathione, might interfere with physiological or behavioral studies if administered *in vivo*. So as part of this purification, we removed GST by cleavage with thrombin. We confirmed that this cleaved mNT5E protein was pure by staining a SDS-PAGE gel for total protein (See Figure 3.1B) and western blotting with an anti-NT5E antibody (See Figure 3.1C). We observed one predominant band at ~62 kDa, corresponding to the calculated molecular weight of unglycosylated mNT5E with a $(His)_6$ tag (61.7 kDa). No additional bands were observed, indicating that mNT5E protein lacked the GST tag and was intact.

3-2 mNT5E is catalytically active

We next used enzyme assays to confirm that our purified protein was catalytically active. Purified recombinant mNT5E protein dephosphorylated AMP with a K_m of 26 µM (See Figure 3.2). This K_m value is within the range of 1-50 µM reported by others using AMP as substrate (Hunsucker et al., 2005; Jörg Servos et al., 1998). Recombinant mNT5E was also inhibited by α,β-methylene-adenosine 5'-diphosphate (α,β-me-ADP; IC_{50}=0.43 µM; See Figure 3.3), a commonly used inhibitor of NT5E (Zimmermann, 1992). For comparison, we found that α,β-me-ADP (0.01-500 µM) did not inhibit recombinant mPAP when AMP was used as substrate (See Figure 3.4). Production of recombinant mPAP was described previously (Sowa et al., 2009). These results suggest α,β-me-ADP has selectivity for NT5E over PAP —an AMP ectonucleotidase that bears no sequence similarity to NT5E.

3-3 mNT5E is Antinociceptive in Naïve Mice

We previously found that a single intrathecal injection of PAP had antinociceptive, antihyperalgesic and antiallodynic effects that lasted for three days and that were dependent on A_1R activation (Sowa et al., 2009; Zylka et al., 2008). To identify an effective dose of mNT5E for *in vivo* studies and to determine if mNT5E also had long-lasting antinociceptive effects, we intrathecally (i.t.) injected wild-type mice with three doses of recombinant mNT5E protein (See Figure 3.5). We then measured noxious thermal and mechanical sensitivity before (baseline, BL) and after mNT5E injection. Six hours after i.t. injection, paw withdrawal latency to the noxious thermal stimulus was significantly increased relative to controls and remained

elevated for two days at all doses tested (See Figure 3.5A). mNT5E did not alter mechanical sensitivity (See Figure 3.5B) nor did it cause paralysis or sedation at any of the doses tested. We similarly found that PAP (from human, cow and mouse) had selective thermal but not mechanical antinociceptive effects in naïve mice and had no obvious motor side effects (Sowa et al., 2009; Zylka et al., 2008).

3-3 mNT5E has long-lasting antinociceptive effects that are A_1R dependent

We next evaluated the antinociceptive effects of mNT5E in the CFA model of inflammatory pain and the SNI model of neuropathic pain. We used wild-type (WT) and $A_1R^{-/-}$ mice to evaluate dependence on A_1R activation and used the contralateral (non-inflamed/non-injured) paw as control. As seen previously by us and others (Sowa et al., 2010; Wu et al., 2005; Zylka et al., 2008), $A_1R^{-/-}$ mice displayed enhanced thermal hyperalgesia after CFA injection and after nerve injury relative to WT mice (See Figure 3.6 A,C). In both chronic pain models, a single i.t. injection of mNT5E had long-lasting thermal antihyperalgesic and mechanical antiallodynic effects in the inflamed/injured paw of WT mice but not A_1R-/- mice (See Figure 3.6A-D). In the CFA model these antinociceptive effects persisted for two days (See Figure 3.6 A,B) whereas in the nerve injury model they persisted for three days (See Figure 3.6 C,D). Consistent with our dose-response study above, mNT5E had thermal but not mechanical antinociceptive effects in the control (non-inflamed/non-injured) paws of WT mice. mNT5E had no antinociceptive effects in $A_1R^{-/-}$ mice, highlighting a critical dependence on A_1R activation. When combined with our biochemical studies and numerous studies of NT5E by others (reviewed by (Colgan

et al., 2006)), our data suggest that all the antinociceptive effects of mNT5E are due to production of adenosine and activation of A_1R (See Figure 3.7). The adenosine made by NT5E (following i.t. injection) could inhibit nociception by acting upon A_1R found on DRG neurons and/or spinal neurons (Reppert et al., 1991; Schulte et al., 2003). Lastly, the fact that NT5E has pronounced antinociceptive effects in live animals places our findings in the most physiologically-relevant context possible.

3-5 Figures

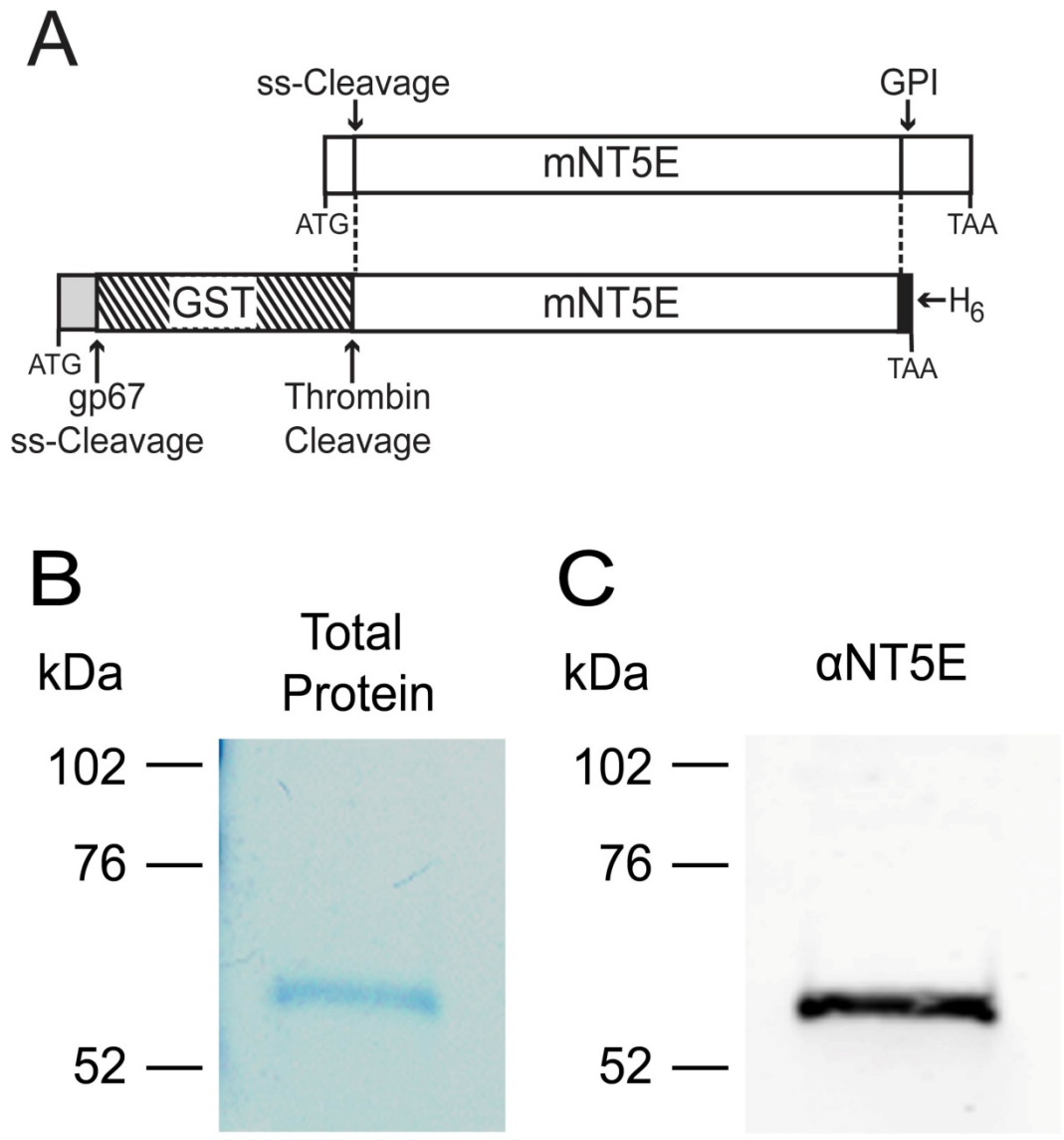

Figure 3.1 Purification of recombinant mNT5E. (A) Diagram of the GST-mNT5E expression construct. (Top) Native mNT5E contains an N-terminal signal peptide (ss-cleavage) and GPI anchor site. (Bottom) The GST-mNT5E fusion construct contains the signal peptide from gp67 of baculovirus *Autographica californica*, GST,

a thrombin cleavage site, the catalytic domain of mNT5E and $(His)_6$ tag. Translation start and stop codons are indicated. (B) GelCode blue-stained SDS-PAGE gel and (C) western blot of purified recombinant mNT5E protein (0.05 µg). The western blot was probed with an anti-mNT5E antibody. (Back to text)

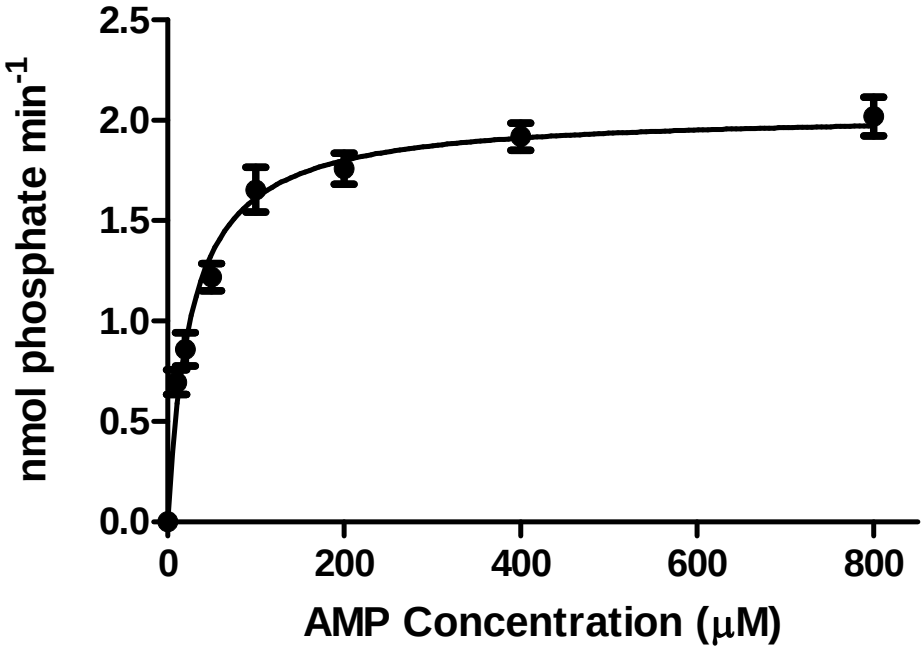

Figure 3.2 mNT5E dephosphorylates AMP. Plot of initial velocity at the indicated concentrations of AMP at pH 7.0. Reactions (n=3 per point) were stopped after 3 min. Inorganic phosphate was measured using malachite green. All data are presented as means ± s.e.m. GraphPad Prism 5.0 was used to generate curve. (Back to text)

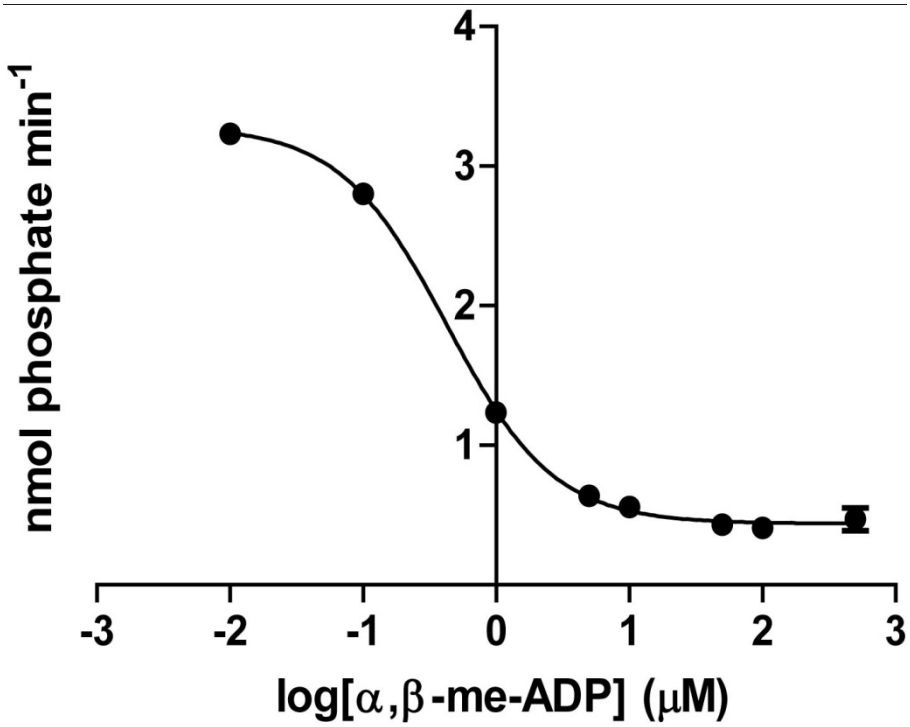

Figure 3.3 Inhibition of mNT5E by α,β-me-ADP. The indicated concentrations of α,β-me-ADP were added to reactions (n=3 per concentration) containing mNT5E (0.07 µg/µL), 100 mM HEPES, pH 7.0 and 400 µM AMP. All data are presented as means ± s.e.m. GraphPad Prism 5.0 was used to generate curve. Error bars are obscured due to their small size. (Back to text)

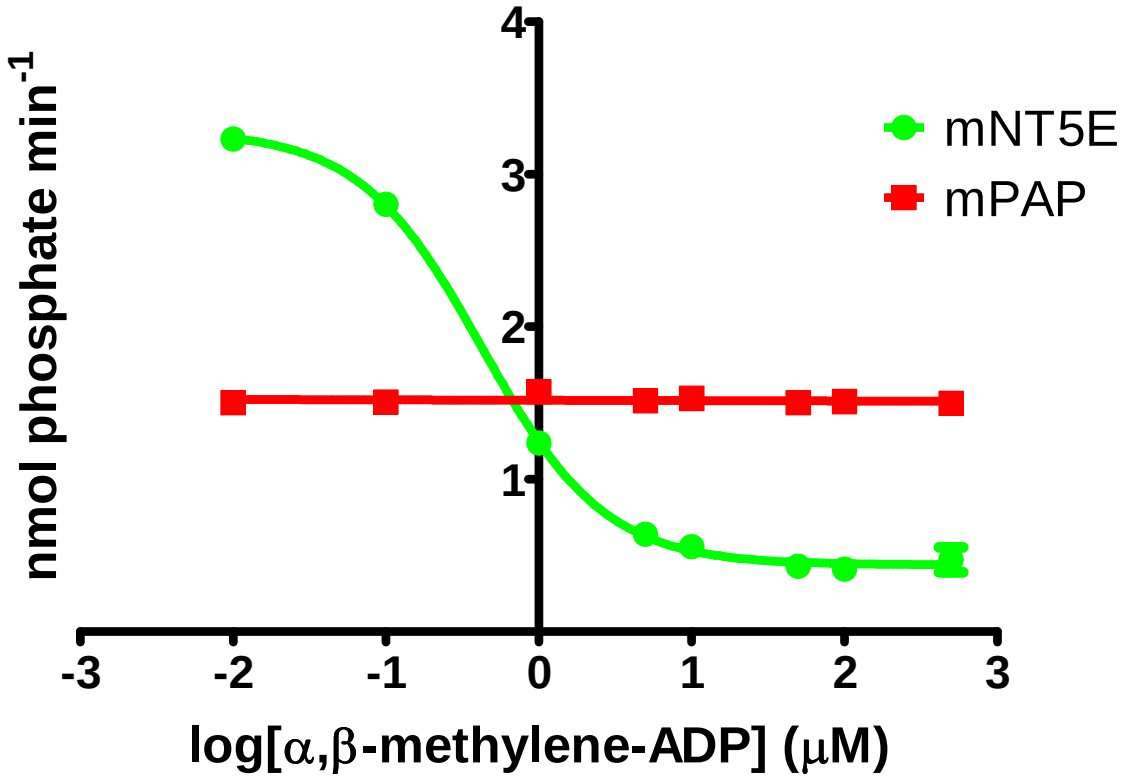

Figure 3.4 mPAP is not inhibited by α,β-me-ADP The indicated concentrations of α,β-me-ADP were added to reactions (n=3 per concentration) containing mNT5E (0.07 µg/µL) or mPAP (0.26 µg/µL), 100 mM HEPES, pH 7.0 and 400 µM AMP. All data are presented as means ± s.e.m. GraphPad Prism 5.0 was used to generate curve. Error bars are obscured due to their small size. (Back to text)

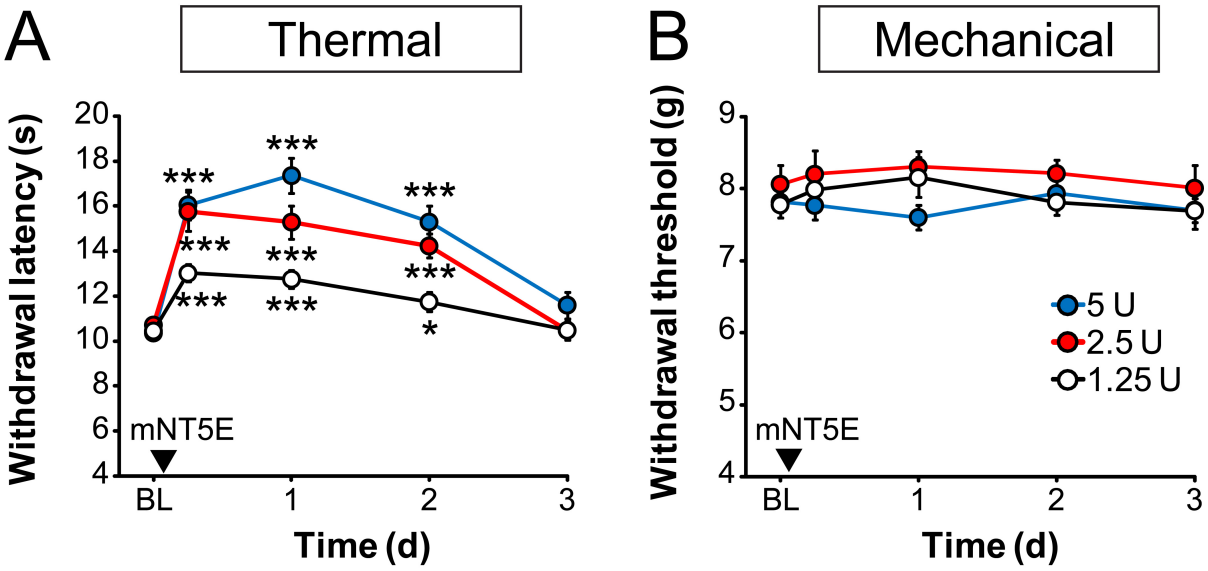

Figure 3.5 Dose-dependent antinociceptive effects of intrathecal mNT5E. Effects of the indicated amounts of mNT5E on (A) paw withdrawal latency to a radiant heat source and (B) paw withdrawal threshold (electronic von Frey apparatus). BL=Baseline. Injection (i.t.) volume was 5 μL. n=10 wild-type mice were used per dose. Paired t-tests were used to compare responses between BL values and later time points for each group. * $P < 0.05$, ** $P < 0.005$; *** $P < 0.0005$. All data are presented as means ± s.e.m. (Back to text)

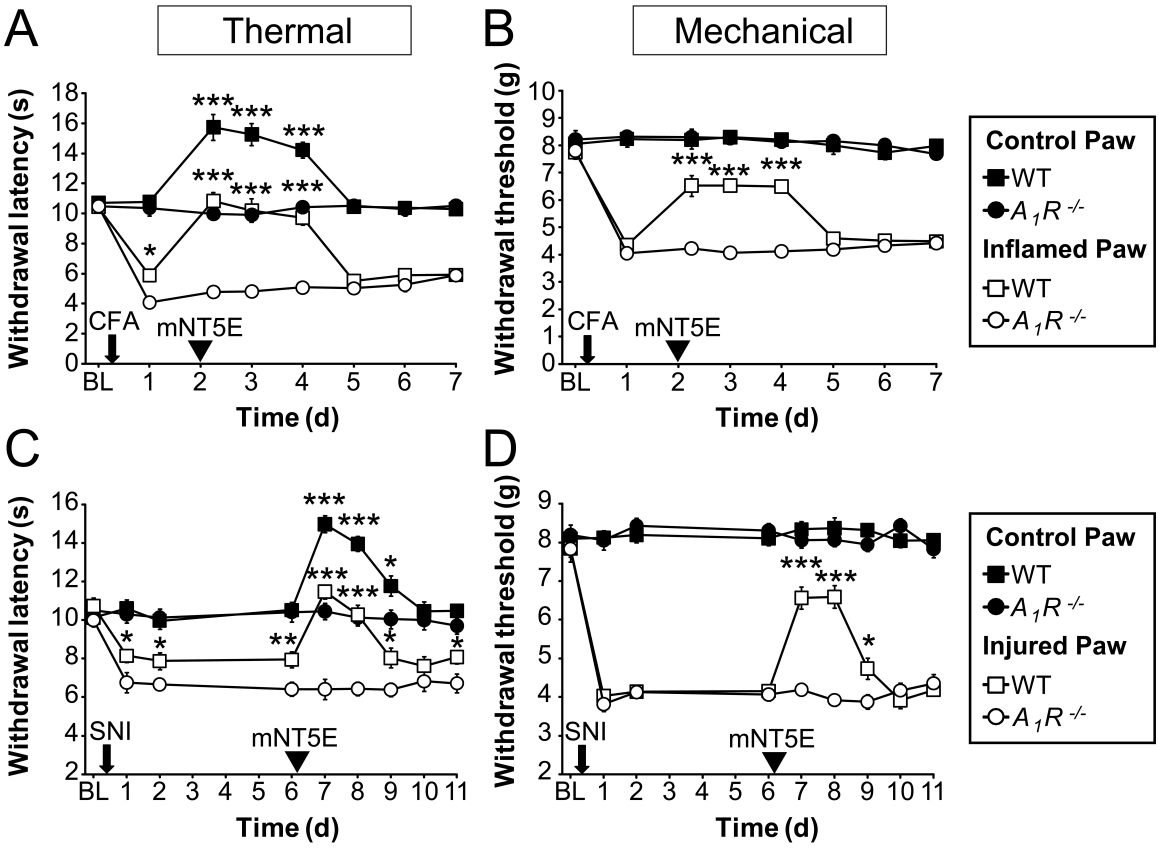

Figure 3.6 mNT5E has antihyperalgesic and antiallodynic effects in WT mice following inflammation and nerve injury. Wild-type (WT) and $A_1R^{-/-}$ mice were tested for (A, C) noxious thermal and (B, D) mechanical sensitivity before (baseline, BL) and after injection of CFA into one hindpaw (A, B; arrow) or following nerve injury (C, D; SNI, arrow). (A, B) One or (C, D) six days later, mNT5E protein (1.7 U) was injected i.t. into all mice (arrowhead) then thermal and mechanical sensitivity was measured for several days. Inflamed/injured and non-inflamed/non-injured (control) hindpaws were tested. Paired t tests 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)

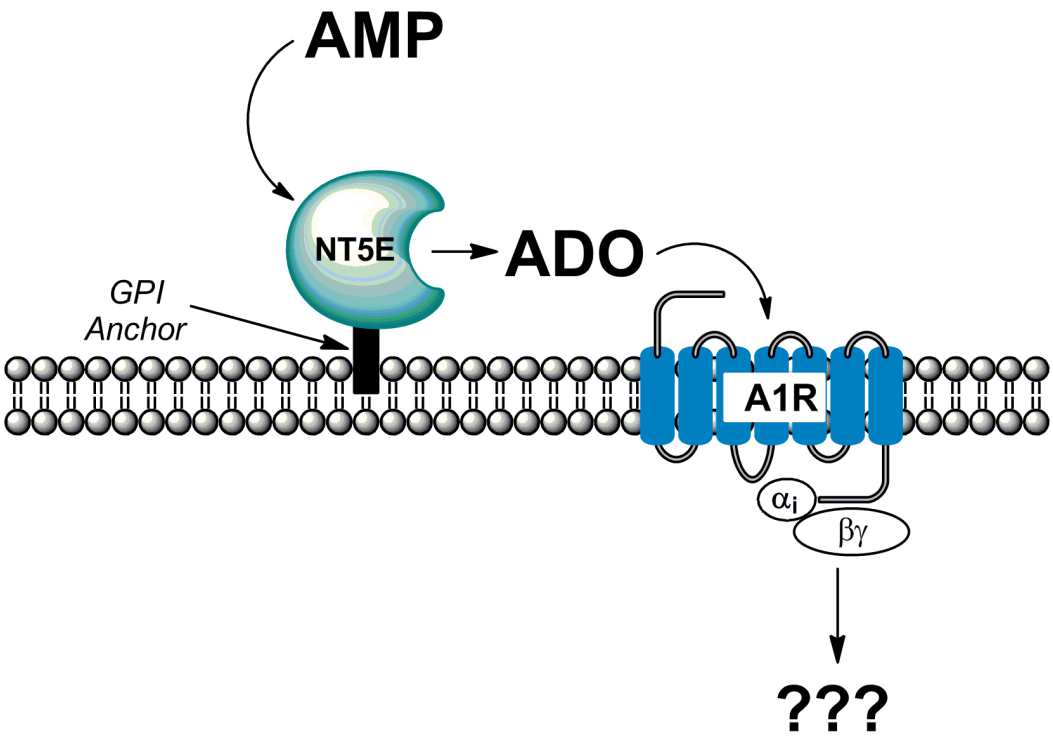

Figure 3.7 mNT5E protein modulates nociception through activation of A_1R receptors. NT5E dephosphorylates AMP in the extracellular space of nociceptive neurons which subsequently leads to the activation of antinociceptive A_1R. Further study is required to determine the mechanism occurring downstream of A_1R activation. (Back to text)

CHAPTER FOUR

DISCUSSION

4-1 NT5E has a Key Role in Nociceptive Circuits

An intriguing dichotomy exists in nociceptive purinergic signaling; purinergic receptors that are activated by nucleotides are associated with increased nociception while receptors that are activated by nucleosides reduce nociception. Pronociceptive receptors are likely activated during injury then deactivated as antinociceptive receptors work against them. Eventually, the injury will heal and both systems will return to baseline. However, if there is an imbalance or malfunction in either system, pathologies could occur that lead to chronic pain. Based on studies in our lab, ectonucleotidases (like NT5E and PAP) serve to restore the balance between the two systems by activating the antinociceptive receptor A_1R.

In our previous study of PAP, there was evidence that other AMP hydrolyzing enzymes besides PAP were present in nociceptive circuits. Subsequently, we found that NT5E, a known ectonucleotidase, is extensively colocalized with PAP in both the DRG and the spinal cord (Sowa et al., 2010). This finding suggested that both enzymes work in conjunction to dephosphorylate AMP in the extracellular space. Following purification of mNT5E, we showed that the protein could dephosphorylate AMP similar to the recombinant mPAP generated by our lab for a prior study (Sowa

et al., 2009). Intrathecal injection of mNT5E into naïve mice produced a long-lasting antinociceptive effect as well as analgesic effects in two models of chronic pain: the CFA model of inflammation and the SNI model of neuropathic pain. Furthermore, we demonstrated that the antinociceptive effects of NT5E are dependent on A_1R since the antinociceptive effect was abolished in $A_1R^{-/-}$ mice.

Prior investigations have shown that spinal injection of adenosine alone produces temporary antinociceptive effects and only when high doses of adenosine are utilized (Belfrage et al., 1999; Sawynok and Sweeney, 1989; Segerdahl et al., 1995; Segerdahl et al., 1994; Sollevi et al., 1995). The half-life of adenosine in the extracellular space of the spinal cord is very short due to the acute activity of nucleoside transporters and metabolic enzymes like adenosine kinase and adenosine deaminase which rapidly remove adenosine from the extracellular environment (Sawynok and Liu, 2003). Therefore adenosine is quickly diverted away from activating antinociceptive adenosine receptors and analgesia is short-lived. Injection of AMP alone has no effect, despite the presence of endogenous ectonucleotidases, for a similar reason (Sowa et al., 2010). Ectonucleotidase-generated adenosine is likely being metabolized much too quickly to show an antinociceptive effect. By pairing AMP with the adenosine kinase inhibitor ITU, thereby increasing the half-life of adenosine, antinociceptive effects were observed (Sowa et al., 2010).

Use of inhibitors for nucleoside transporters and adenosine metabolic enzymes has served to increase the antinociceptive effects of adenosine (Jarvis et al., 2002b; Kowaluk and Jarvis, 2000; Lavand'homme and Eisenach, 1999). Yet

none of these inhibitors match the potency or last nearly as long as NT5E or PAP protein. Antinociceptive effects can also be produced with specific agonists for A_1R, such as CPA (Curros-Criado and Herrero, 2005; Reeve and Dickenson, 1995). However, the effects of these agonists are also short-lived. Additionally, these agonists produce profound motor side effects such as paralysis (Zylka et al., 2008). These side effects likely occur due to the overt and nonspecific activation of A_1R on motor neurons as well as nociceptors. As such, NT5E and PAP provide a means for activating A_1R without observable side effects in rodents and the endurance of this A_1R-mediated analgesic effect is most likely due to the remarkably long half-life of these enzymes. Additional studies will have to be conducted to determine the exact mechanism behind the enduring analgesia of NT5E and PAP. Nevertheless, our studies have firmly established NT5E as a key regulator of purinergic signaling in nociceptive circuits.

4-2 NT5E is well-positioned to modulate adenosine receptors

Our study shows that the analgesic and antinociceptive effects of NT5E are dependent on A_1R. Overlap between A_1R-expressing and NT5E expressing neurons occurs in lamina II of the dorsal horn (Schulte et al., 2003). A_1R is concentrated near nonpeptidergic terminals in the dorsal horn, but are not in close contact with terminals from peptidergic neurons— which project to lamina I. A_1R has also been found on the presynaptic terminals of small and medium diameter nociceptive neurons (Schulte et al., 2003). Additionally, multiple groups have found that activation of A_1R inhibits presynaptic release of glutamate from unmyelinated

terminals and also inhibits the activity of postsynaptic neurons in lamina II (Lao et al., 2001; Li and Perl, 1994; Patel et al., 2001). Given that NT5E is expressed in the nonpeptidergic neurons that are near A_1R-expressing neurons, NT5E is in an optimal position to interact with these receptors.

Another possibility that has yet to be fully tested is the possible interaction of NT5E-generated adenosine with other adenosine receptors. While A_{2B} and A_3 receptors are primarily localized to the periphery, A_{2A}R can be found in DRG neurons, glial cells and the spinal cord (Bura et al., 2008; Cunha et al., 2006; Haskó et al., 2005; Hussey et al., 2007; Kaelin-Lang et al., 1998). Debate remains about the presence of A_{2A}R in the mouse spinal cord. One study which utilized quantitative audioradiography reported that there was no A_{2A}R in the spinal cord (Bailey et al., 2002). Yet, a more recent study claims that A_{2A}R expression can be detected in wild type mice with RT-PCR (Bura et al., 2008). Nevertheless, due to the lack of a specific antibody no study has pinpointed the precise location of A_{2A}R in the mouse spinal cord or DRG.

NT5E most likely does not lead to A_{2A}R activation *in vivo*. A recent study conducted by Loram, et al. has shown that antinociceptive effects can be achieved in rats through intrathecal injection of specific A_{2A}R agonists. These effects lasted for weeks following a single injection and proved to be analgesic in a model of drug-induced allodynia. Though this study has yet to be replicated in mice, the fact that these effects lasted much longer than effects generated with mNT5E protein suggests that A_{2A}R is not being activated by NT5E. Most likely, NT5E, PAP and

$A_{2A}R$ are differentially localized, preventing interaction between the ectonucleotidases and $A_{2A}R$. Additional *in vivo* studies with PAP and NT5E in $A_{2A}R^{-/-}$ mice have to be conducted to reach a final conclusion.

4-3 Determining the mechanism behind mNT5E antinociception

While mNT5E recombinant protein produces antinociceptive effects that are similar to PAP in live animals, the mechanism behind mNT5E's effects has yet to be determined beyond A_1R (See Figure 3.7). One approach to determining the mechanism would be to develop a cell based assay using culture cells *in vitro*. A_1R activation has been shown to inhibit adenylate cyclase, decrease levels of cAMP, decrease PKA activity and also activate PKC (Bhave et al., 2003; Bhave, et al., 2002; Huang et al., 2006). Different inhibitors and activators could then be used to determine which pathways are being triggered or suppressed by mNT5E. Additional work could be done *in vivo* if applicable.

4-4 Using mNT5E as a tool for studying adenosine receptor signaling

Whereas PAP is expressed primarily in the prostate, the spinal cord and the gut, NT5E is found in numerous tissues from macrophages to the lungs (Zimmermann, 1992). This near-ubiquitous expression of NT5E suggests that NT5E is intimately connected to adenosine signaling in multiple systems. Therefore, mNT5E protein and its ability to rapidly and specifically deplete AMP could be a useful tool for studying the role of adenosine receptor signaling in various physiological processes.

Adenosine has been connected to cilia beating frequency in the lungs (Lazarowski et al., 2004; Picher et al., 2003; Rollins et al., 2008). The movement of the cilia is essential for clearing mucus from the lungs and a malfunction in cilia movement could lead to insufficient defense against infectious lung diseases. A prior study has shown that NT5E as well as alkaline phosphatase is responsible for hydrolyzing AMP in lung tissue (Picher et al., 2003). mNT5E protein could be used to definitively show that NT5E is required for regulating cilia movement. The protein might also be potentially useful in future treatments for disorders like Cystic Fibrosis where controlling cilia beat frequency might help clear troublesome mucus.

mNT5E could also be used to further study the role of purinergic signaling in sleep. Caffeine is a well-known stimulant that antagonizes both A_1 and A_{2A} adenosine receptors. Most adults in this country start their days by blocking out adenosine with a cup of coffee which is a testament to the importance of adenosine in regulating sleep (Basheer et al., 2004). And given the wide expression of NT5E in the brain, it is likely that NT5E is an important player in the regulation of sleep. Studies have shown that adenosine levels fluctuate in different parts of the brain between consciousness and sleep (Huston et al., 1996; Murillo-Rodriguez et al., 2004; Porkka-Heiskanen et al., 1997). Adenosine is also critical for regulating the depth of and length of sleep (Landolt, 2008; Porkka-Heiskanen et al., 1997). Blocking adenosine metabolism leads to prolonged sleep and more intense non-REM sleep (Okada et al., 2003; Radek et al., 2004; Radulovacki et al., 1983). Given that sleep studies have yet to be conducted on *Nt5e*$^{-/-}$ or *Pap*$^{-/-}$ mice, the role of ectonucleotidases in sleep regulation remains unclear. mNT5E could be a useful

tool in sleep studies and may demonstrate therapeutic value for treating sleep disorders.

In addition to sleep and lung function, adenosine has a part in regulating vascular functions pertaining to ischemia. During stress or injury, the cardiovascular system releases adenosine which then acts on receptors to promote cell survival (Eltzschig et al., 2003). Call ischemic preconditioning, this cardioprotective process can reduce the size of myocardial infarctions (Eckle et al., 2007b; Headrick et al., 2003). Expression of NT5E increases during this process and is the most likely source of adenosine production (Eckle et al., 2007a; Headrick et al., 2003; Kitakaze et al., 1999). *In vivo* studies using snake venom failed to stimulate ischemic preconditioning using purified 5' nucleotidase (Eckle et al., 2007b). The lack of effect may be a species-specific effect. Therefore it remains possible that mNT5E is cardioprotective. Additionally, the toxicity concerns inherent with using snake venom proteins would be eliminated by using recombinant mNT5E.

Response to hypoxia is another system that is closely controlled by adenosine. Levels of adenosine rise in the CNS following hypoxia (Koos et al., 1997). This increase has been connected to hypoxia-inducible factor-1, a protein that mediates upregulation of NT5E (Ledoux et al., 2003; Synnestvedt et al., 2002; Thompson et al., 2004). As such, mNT5E protein could be used to protect against hypoxic damage. A study which used snake venom 5'-nucleotidase showed that the protein could reduce vascular permeability in wildtype mice under hypoxic conditions thereby reducing the severity of the pulmonary edema that the animals suffered (Thompson et al., 2004). Mammalian NT5E would likely perform just as well or

better than the snake venom protein, making mNT5E a useful protein for studying the purinergic signaling associated with hypoxia.

Adenosine signaling has also been tied to inflammation. While the role of central nervous system $A_{2A}R$ in nociception remains unclear, there is no doubt that these receptors have anti-inflammatory properties in immune cells (Sitkovsky et al., 2004). Activation of these receptors ultimately leads to the inhibition of cytokine production as well as promoting an increase in anti-inflammatory cytokines (Erdmann et al., 2005; Jacobson and Gao, 2006; Lappas et al., 2005; Sitkovsky et al., 2004). NT5E, like the $A_{2A}R$, is expressed extensively in the immune system. A few studies have shown that NT5E-generated adenosine affects inflammation, but these studies were dependent on either genetic manipulation or indirect measurement methods to reach their conclusions (Cronstein, 2005; Eltzschig et al., 2004; Morabito et al., 1998). Therefore, mNT5E protein could be utilized in inflammatory studies to determine whether NT5E affects inflammation. In the future, the protein might also be a useful anti-inflammatory agent for treating immune disorders and chronic inflammatory conditions like arthritis.

4-5 Exploring the Combined Roles of NT5E and PAP

Though our lab has provided evidence that NT5E and PAP are antinociceptive ectonucleotidases that function through the A_1R, the question of how these two enzymes operate together under physiological conditions remains. Considering that these two enzymes with similar mechanisms of action are

extensively colocalized, it is logical to conclude that there is functional redundancy. But why would such a redundancy evolve in mammals?

One possibility is that the enzymes only function under specific conditions. NT5E performs optimally at a neutral pH and exclusively hydrolyzes 5'-monophosphates, whereas PAP can function well within a pH range of 4-8 and can dephosphorylate different substrates (including ADP and ATP, though AMP remains the favored substrate) (Silverman and Kruger, 1988a; Van Etten, 1982; Zimmermann, 1992). Additionally the average K_m value for AMP is a thousand-fold smaller for NT5E than it is for PAP (Sowa et al., 2009; Zimmermann, 1992). Therefore NT5E and PAP may be capable of performing the same functions, but under different conditions. In normal conditions with a neutral pH environment, NT5E might have a better affinity for AMP than PAP. Injury or inflammation, however, could lead to changes in extracellular pH that decreases NT5E's affinity for AMP. In this situation, PAP would still be able to function and mediate analgesia through A_1R until a neutral pH is restored.

The dual influence of NT5E and PAP also has yet to be extensively studied in the peripheral nervous system. Our lab has found both PAP and NT5E on nociceptive axon terminals in the epidermis as well as cells in the superficial dermis and keratinocytes (Sowa et al., 2010; Zylka et al., 2008). All four adenosine receptor subtypes are found in skin keratinocytes, yet it is not known whether NT5E or PAP interact with these receptors (Burnstock, 2009). The fact that all the knockout animals for NT5E, PAP and A_1R show increased sensitivity to mechanical stimulation of their paws following injury indicates that adenosine signaling is

important for nociception. Yet, studies of the peripheral actions of purified PAP and NT5E proteins will have to be conducted to determine if these ectonucleotidases are capable of promoting analgesia through peripheral nerve endings.

4-6 Ectonucleotidases and the Future of Pain Treatment

The discovery of antinociceptive ectonucleotidases in our lab provides exciting new possibilities for pain therapy. Since these enzymes are endogenous to the nervous system, they are unlikely to produce toxic side effects and are less likely to generate complications with the body's innate defenses. An additional benefit of treating pain with these enzymes is that no observable side effects, such as paralysis, have appeared in our animal studies. This is potentially a distinct advantage over treating pain with adenosine alone as non-specific signaling can occur, leading to activation of receptors besides A_1R. The problem of non-specific signaling has been noted in clinical trials where adenosine treatment in humans led to headaches, back pain and other undesirable side effects (Belfrage et al., 1999; Eisenach et al., 2002; Lavand'homme and Eisenach, 1999; Segerdahl et al., 1995; Sollevi et al., 1995). Direct activation of A_1R also presents a problem as many agonists have motor side effects (Sawynok, 1998; Zylka et al., 2008). Therefore the indirect activation of A_1R through these ectonucleotidases presents a multitude of potential treatment options.

One fairly obvious treatment method would be to inject recombinant NT5E or PAP protein into patients. Since both proteins have been successfully purified, this is one of the most feasible approaches for treating patients with intractable pain. The

drawback of treating patients with pure protein is that so far our studies have only shown that purified PAP and NT5E are effective when injected intrathecally. This delivery method would be beneficial to patients with the most severe debilitating pain conditions or could be used to deliver the enzyme for patients undergoing surgery, but would not ultimately be practical for most patients who suffer from chronic pain. The proteins would be digested if they were delivered orally. As such, one approach would be to search for inhibitors and activators of NT5E and PAP that could possibly be delivered orally or through intravenous injection.

A full study of the side effects of NT5E and PAP in animals also has yet to be completed. Though we have not seen any severe side effects in the animals we have tested, there may be more subtle side effects that cannot be detected by merely looking at the animals. Cardiac function and body temperature could be measured following PAP or NT5E treatment to determine if there are any adverse effects on these systems. Motor function could also be more thoroughly assessed through tests such as the rotorod, the grip test, and gait measurement. Given the importance of adenosine in regulating sleep, treated animals might also be examined for any irregularities in their sleep routine. The effect of these peptides on cognition, emotions and concentration cannot be thoroughly assessed through animal testing. Nevertheless, all possible side effects that can be tested in the animal model will have to be evaluated.

An additional issue that remains to be addressed by our studies is the effectiveness of our enzymes in female animals. In humans, a disproportionate number of women are reported to suffer from chronic pain conditions compared to

men (Foundation, 2009). Numerous studies have explored this disparity between the genders by using animal models and there is a growing body of evidence that gonadal hormones affect nociception (Craft et al., 2004). Despite a recommendation by the International Association for Studying Pain that all researchers conduct their behavioral tests with both sexes, or *only* in females if there are budget constraints, the majority of pain studies continue to be conducted solely in male animals (Greenspan et al., 2007). Whether gender testing is the responsibility of the primary researcher or the companies which prepare treatments for use in humans is still open for debate. Regardless, new insights may be gained by testing the effectiveness of NT5E and PAP proteins in female animals.

A great deal of hard work remains to translate our ectonucleotidase-based treatments from rodents to human patients. In the face of a growing pain epidemic, these challenges are worth undertaking. Pain afflictions are debilitating not only to the individuals who endure them, but also inflict great damage, both financial and emotional, on our society at large. Our findings have the potential to provide new options to the millions of people nationwide that suffer from chronic pain.

APPENDIX I

Acph1: A Catalytically Active Ortholog of PAP in *Drosophila melanogaster*

Summary

Our lab has successfully demonstrated that spinal injection of secreted PAP produces antinociceptive in mice. Nevertheless, using a mouse model has limitations that make it difficult to identify proteins that functionally and genetically interact with PAP. An ortholog of PAP in *Drosophila melanogaster*, called *Acph1* (dmPAP), which shares sequence and biochemical similarities to mouse and human PAP was identified in prior studies (Chung et al., 1996; MacIntyre, 1966). To functionally test dmPAP *in vitro*, we constructed expression clones of dmPAP fused to the yellow fluorescent protein Venus and the red fluorescent protein mCherry. dmPAP possessed a similar perinuclear and membrane localization to mouse PAP when expressed in cultured Rat1 cells (Figure A1.1). Histochemical staining in HEK 293 cells using adenosine monophosphate (AMP) as a substrate also revealed staining patterns similar to mouse and human PAP (Figure A1.2). A fluorometric assay and additional histochemical staining showed that dmPAP could be inhibited by L(+)-tartrate, a known inhibitor of mammalian PAP (Figure A1.3 and Figure A1.4). These results suggested that dmPAP has the same enzymatic properties as the mouse and human versions. However, studies of calcium responses in dmPAP transfected cells were inconclusive and embryonic in situ hybridization of dmPAP in wildtype embryos also provided no firm evidence that dmPAP is present in the nervous system of *Drosophila melanogaster* (data not shown and Figure A1.5). As

such we concluded that there were not enough similarities between the fly model and our current mouse model to pursue genetic studies in fruit flies.

Methods

Molecular Biology of the dmPAP construct

The dmPAP expression plasmid was generated by PCR amplification of nt 185-1498 (from GenBank accession # NM_080178.4) using Phusion polymerase and the fly cDNA construct RE14694 (DRGC, Indiana University) as a template. N-terminal primer: 5'-cgctctagaaccatgtggaaccacccaagccag C-terminal primer: 5'-cgccggccggagccatttgagagtacgagg. The EagI site in the C terminal primer sequence (underlined) was used to link the dmPAP to an expression sequence for the fluorescent protein Cherry. Then Xb I site (underlined) in the N-terminal primer sequence and another Xb I site located at the C-terminal end of the mCherry sequence were used to facilitate cloning the dmPAP sequence plus mCherry into the vector pcDNA3.1. The final plasmid contained an expression sequence for dmPAP and mCherry and was sequence verified.

Cell Transfection

Constructs were transfected into HEK293 cells or Rat1 cells using Lipofectamine (18324-012, Invitrogen) and Plus Reagent (11514-015, Invitrogen) according to the included protocol. Cells were incubated in the transfection reagents for at least three hours in serum free media then replaced with serum-containing media for

overnight incubation. Expression of fluorescent constructs was confirmed with a Zeiss Axioskop.

Cell Histochemistry

Enzyme histochemistry was performed essentially as described by Shields et al., with modifications suggested by Silverman and Kruger (Shields et al., 2003a; Silverman and Kruger, 1988b). Briefly, cells 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℃ for 2 hr in TM b uffer containing 8% sucrose (w/v), 1 mM adenosine monophosphate , and 2.4 mM lead nitrate. Lead nitrate was 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). Images were acquired using a Zeiss Axioskop and an Olympus DP-71 camera.

Fluorometric Assay

Cultured HEK293 cells transfected with dmPAP were lysed using ice cold RIPA Lysis Buffer (1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS dissolved in 1X PBS). Protease inhibitors (a single Mini Complete Tablet, 11836153001, Roche) were added to the buffer before lysis. Twenty-five microliters of 100 mM sodium acetate (+/-) L(+)-tartrate was added to 25 µL cell lysate along with 50 µL of 200 mM DiFMUP substrate (from Enzchek phosphatase assay system, E6646, Invitrogen).

Flourescence was measured with excitation set at 390 nm and emission detection at 510 nm on a Fluorscan Ascent FL plate reader (Thermosystems).

Embryonic In Situ Hybridization

Fly embryos were collected from grape-juice agarose plates. Embryos were dechorionated for 5 min with a 50% bleach solution then rocked for 25 min in a 50% heptane, 5% paraformaldehyde(PFA) in 1X PBS gradient solution to fix the embryos. Following fixation, the fixative layer of the solution was removed and methanol was added to remove the vitallin membrane. Embryos were stored in 100% methanol at -20℃. Embryos were rehydrated in a 3:1, methanol: 4% PFA in 1X PBS solution for 2 min then in a 1:3, methanol: 4% PFA in 1X PBS solution for 5 min. Afterwards, embryos were washed six times with a 0.1% Tween 20, 1X PBS solution. Then embryos were rocked in hybridization buffer (4X SSC, 50% deionized formamide, 0.01% Tween 20) for 1 hr at room temperature. In the meantime, an *Acph1* anti-sense probe was diluted 1:100 in hybridization buffer with 5% dextran sulfate. Dilution was heated at 85℃ for 5 mins, placed on ice f or 2 mins then heated at 55℃ for 2 mins. Hybridization buffer was removed from the embryos and replaced with the warmed probe. Embryos were incubated in probe overnight at 55℃. Next day the embryos were washed in wash buffer (2X SSC, 50% formamide, 0.1% Tween 20) for 30 min intervals eight times. Then embryos were incubated overnight in wash buffer at 55℃. Next day the embryos were rinsed in 0.1% Tween 20, 1X PBS solution then rocked for 30 min at room temperature in the same solution. An

antibody solution containing 5% goat serum and a 1:2000 dilution of anti-DIG-AP fab fragments (Roche) was added to the embryos and embryos were rocked for 2 hr at room temperature. Embryos were rinsed twice with 0.1% Tween 20, 1X PBS solution then washed nine times with the same solution at 10 min per wash. Then the embryos were rinsed two times with fresh made AP buffer (50 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris pH 9.5, 0.01% Tween 20). Afterwards embryos were treated with BCIP/NBT color solution (3.5 µL 50 mg/mL BCIP and 4.5 µL NBT in 1 mL AP buffer) until desired color was achieved. Embryos were washed three times in with 0.1% Tween 20, 1X PBS solution to stop the color reaction then mounted on slides with 70% glycerol.

Figures

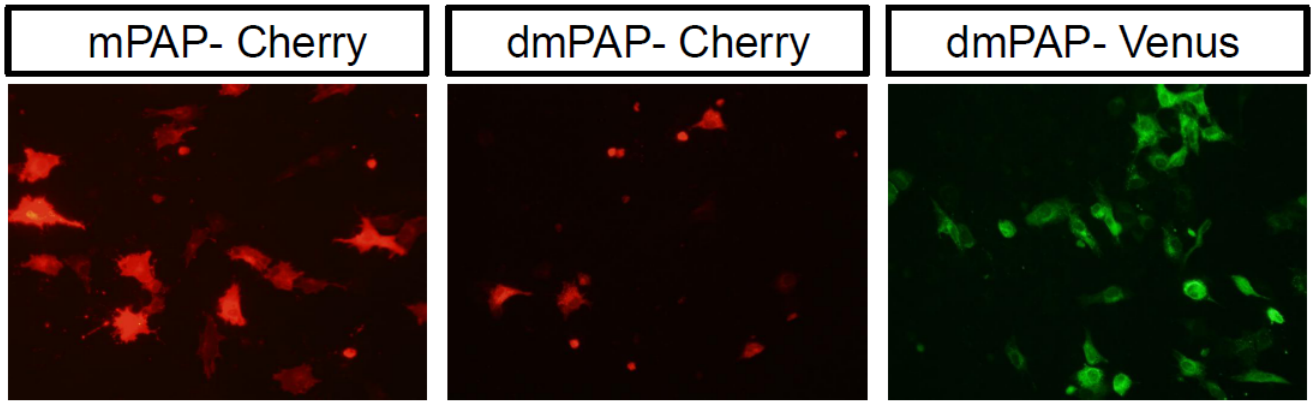

Figure A1.1 dmPAP and mPAP have similar localization in cultured cells. Cultured Rat1 cells were transfected with fluorescently tagged constructs for dmPAP and mPAP. Fluorescence from both dmPAP constructs appears in the membrane and the perinuclear space. This resembles the fluorescence in mPAP transfected cells, thereby suggesting a similar localization of mPAP and dmPAP.

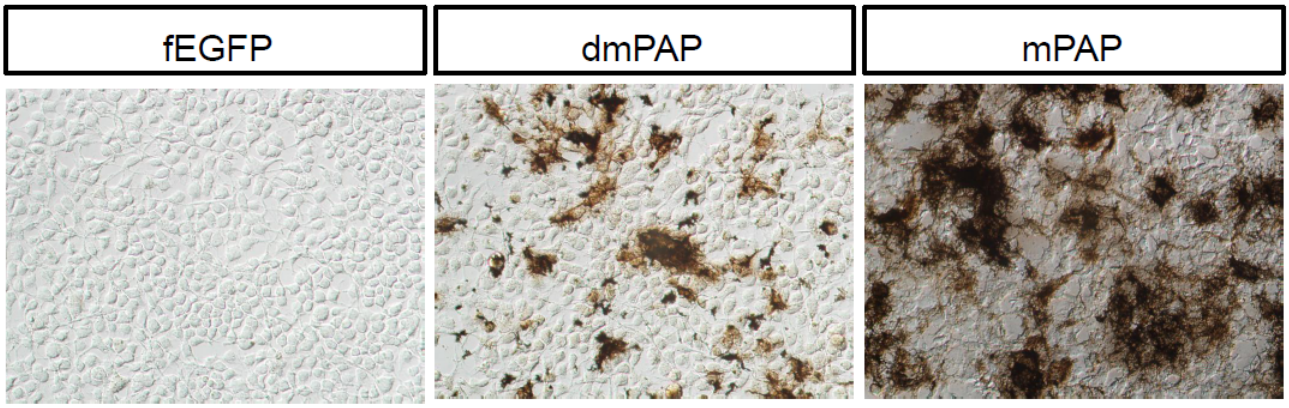

Figure A1.2 dmPAP dephosphorylates AMP. Cultured HEK 293 cells were transfected either with fEGFP, a cherry tagged dmPAP construct or a cherry tagged mPAP construct. Lead histochemistry was performed at pH 5.6 using 1 mM adenosine monophosphate (AMP) as a substrate. Cells transfected with dmPAP had near identical staining patterns to mPAP.

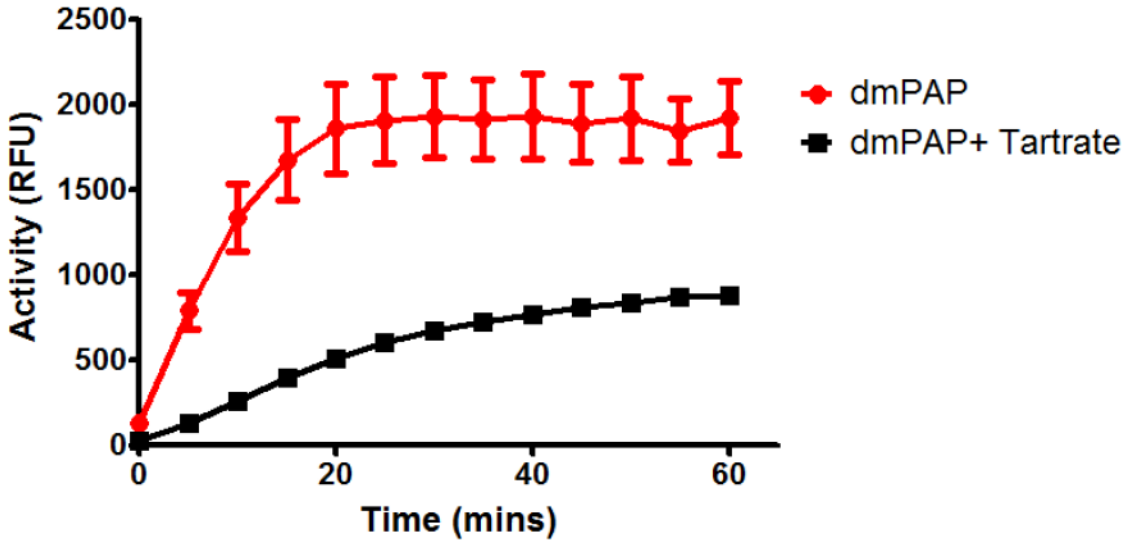

Figure A1.3 dmPAP is inhibited by L(+) Tartrate. HEK 293 cells were transfected with dmPAP and lysed using RIPA lysis buffer. Cell lysates were tested (w/ and w/o)

tartrate in a fluorometric assay for acid phosphatase activity using DiFMUP as a substrate. Activity was reduced by half when lysates were treated with tartrate.

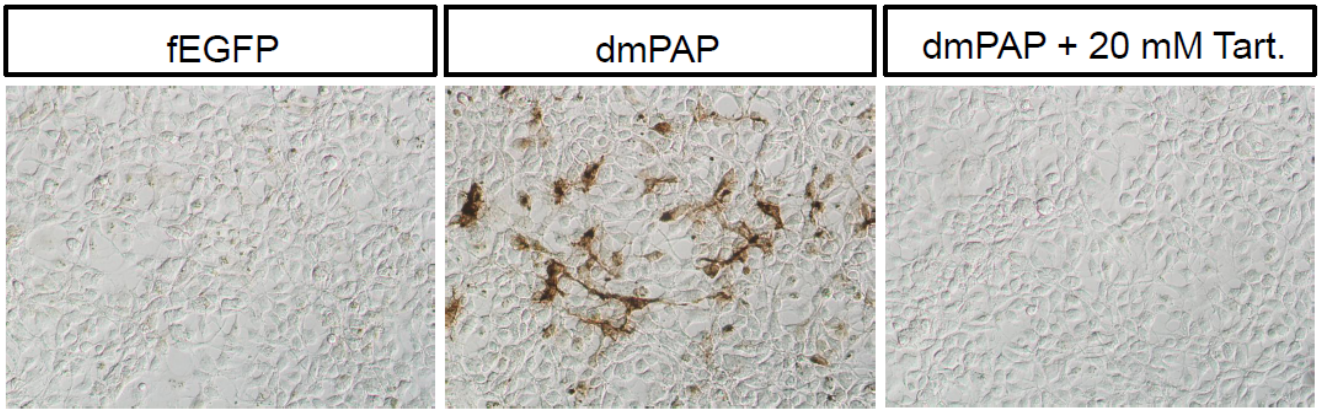

Figure A1.4 dmPAP dephosphorylation of AMP is inhibited by L(+) tartrate. HEK293 cells were transfected with either fEGFP or dmPAP. Lead histochemistry was performed (w/ and w/o) tartrate at pH 5.6 using 1 mM AMP as a substrate. AMP staining pattern was eliminated in tartrate treated cells, suggesting that tartrate inhibited dmPAP dephosphorylation of AMP.

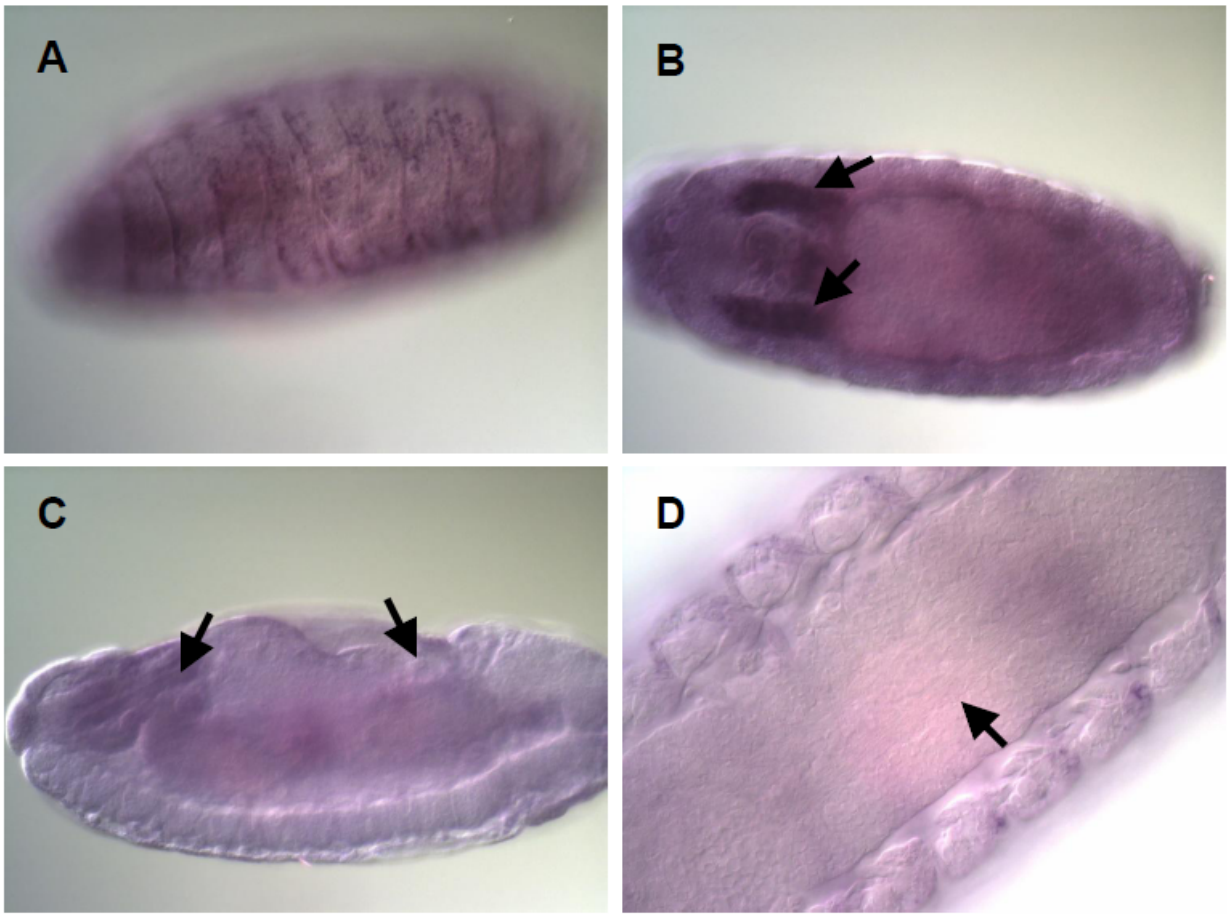

Figure A1.5 dmPAP has analogous expression to mPAP in *D. melanogaster* embryos aside from the CNS. In-situ hybridization of wildtype fly embryos for dmPAP revealed expression in (A) the epidermis, (B) the salivary glands, and (C) the gut. Staining was either absent or undetectable in the CNS (D). PAP is also expressed in skin, salivary glands, and the gut in mammals, yet the lack of prominent CNS staining led us to determine that the fly model would not be useful for conducting genetic studies that involve nociception.

APPENDIX II

PAP is Capable of Dephosphorylating Benfotiamine

Summary

Research has shown that B vitamins, especially thiamine, are required for proper nervous system function (Itokawa, 1996). Deficiencies in thiamine lead to a condition called Beriberi which is characterized by fatigue, loss of motor function and widespread chronic pain (Lonsdale, 2006). On the other hand, thiamine treatment has been shown to be analgesic in animal models of pain (Caram-Salas et al., 2006; França et al., 2001; Jolivalt et al., 2009; Song et al., 2009). High doses of thiamine, however, are required to see this effect. Prior investigators proposed that the water solubility of thiamine leads to a small rate of absorption. As such, benfotiamine, a lipid-soluble derivative of thiamine was synthesized in the early 1960s. Benfotiamine has been shown to be analgesic in models of neuropathic pain and inflammatory pain in both humans and rodents (Jolivalt et al., 2009; Sánchez-Ramírez et al., 2006; Simeonov et al., 1997; Stracke et al., 1996). Furthermore, our lab has shown that when benfotiamine is antinociceptive when injected into naïve animals (Coleman, et al., unpublished data). Currently, the mechanism behind benfotiamine's analgesic effects is unknown. Our lab has recently discovered that benfotiamine antinociceptive effects are ablated in *Pap*$^{-/-}$ mice. These data suggest that PAP is a key factor in the antinociceptive effects of benfotiamine. Here we show that PAP has the capability to dephosphorylate benfotiamine in two separate

assays (Figure A2.1 and Figure A2.2). Additionally, we show that PAP has a better affinity for dephosphorylating benfotiamine in an acidic environment than a generic alkaline phosphatase—another class of ectonucleotidases found in the spinal cord (Figure A2.3 and Table A2.1). These findings confirm that PAP is capable of dephosphorylating benfotiamine *in vitro*.

Methods

Enzymatic reactions (50 μL final) were carried out with recombinant mNT5E at 37℃ for 3 minutes in 100 mM sodium acetate for the pH 5.6 reactions, 100 mM HEPES for the pH 7.0 reactions, and 100 mM Tris for pH 8.5 reactions with adenosine 5'-monophosphate (AMP disodium salt, Fluka, 01930) 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 incubated 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). 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 ℃ then d iluted in 0.9% saline to a concentration of 0.43 μg/μL (0.02 U/ μL). Recombinant bovine alkaline phosphatase was purchased from Sigma (P8361, expressed in Pichia pastoris, >4000 U/mg protein) and was diluted to 2.1 μg/μL in 0.9% saline (0.5 U/ μL). One microliter of enzyme was used for each reaction.

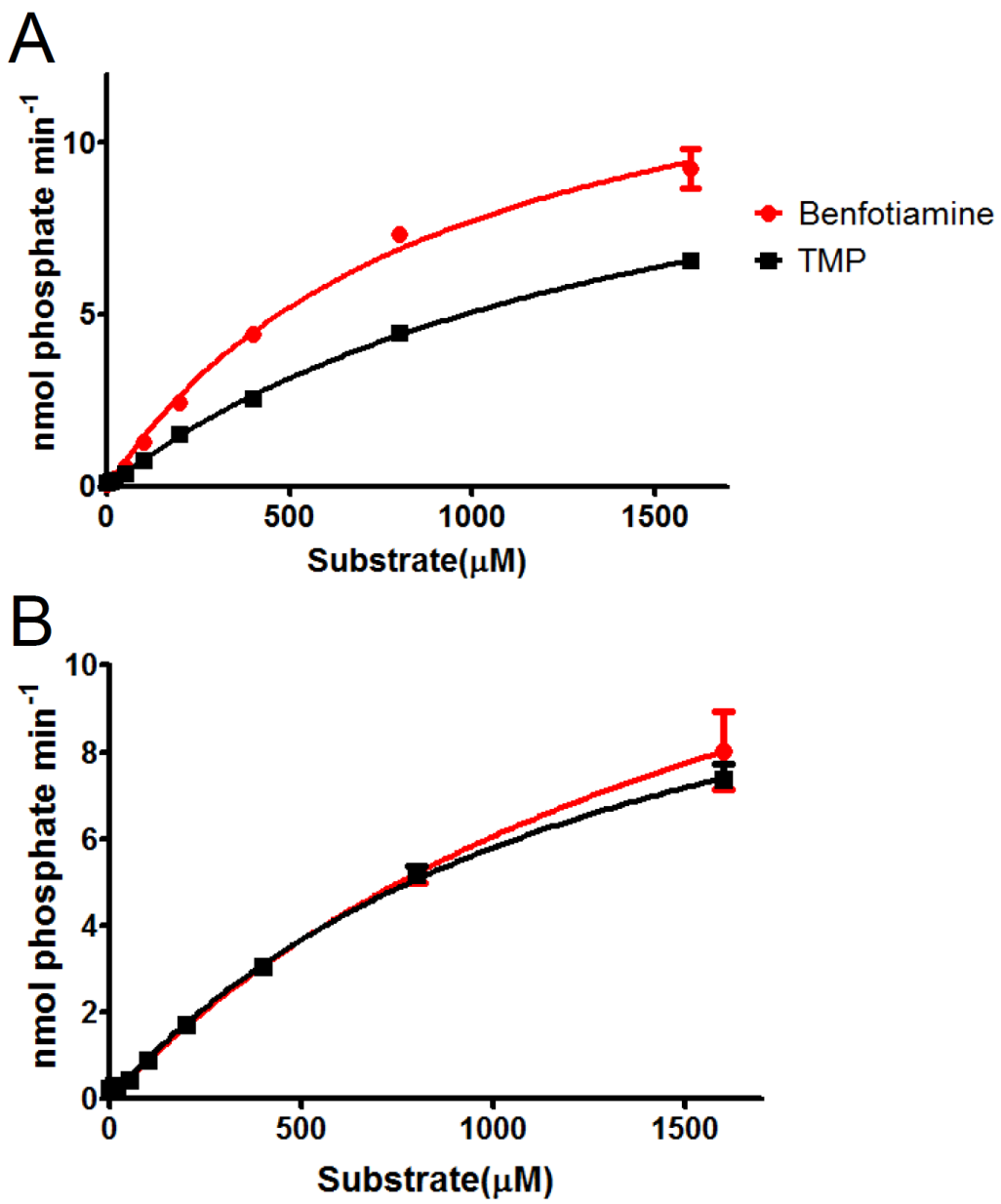

Figure A2.1 Human PAP dephosphorylates Benfotiamine in a malachite green assay. Plot of initial velocity at the indicated concentrations of TMP or benfotiamine at (A) pH 5.6 and (B) pH 7.0. K_m values at pH 5.6 are 354.4 µM and 101.6 µM for TMP and benfotiamine respectively. K_m values at pH 7.0 are 1361 µM and 1865 µM respectively. 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. GraphPad Prism 5.0 was used to generate curves.

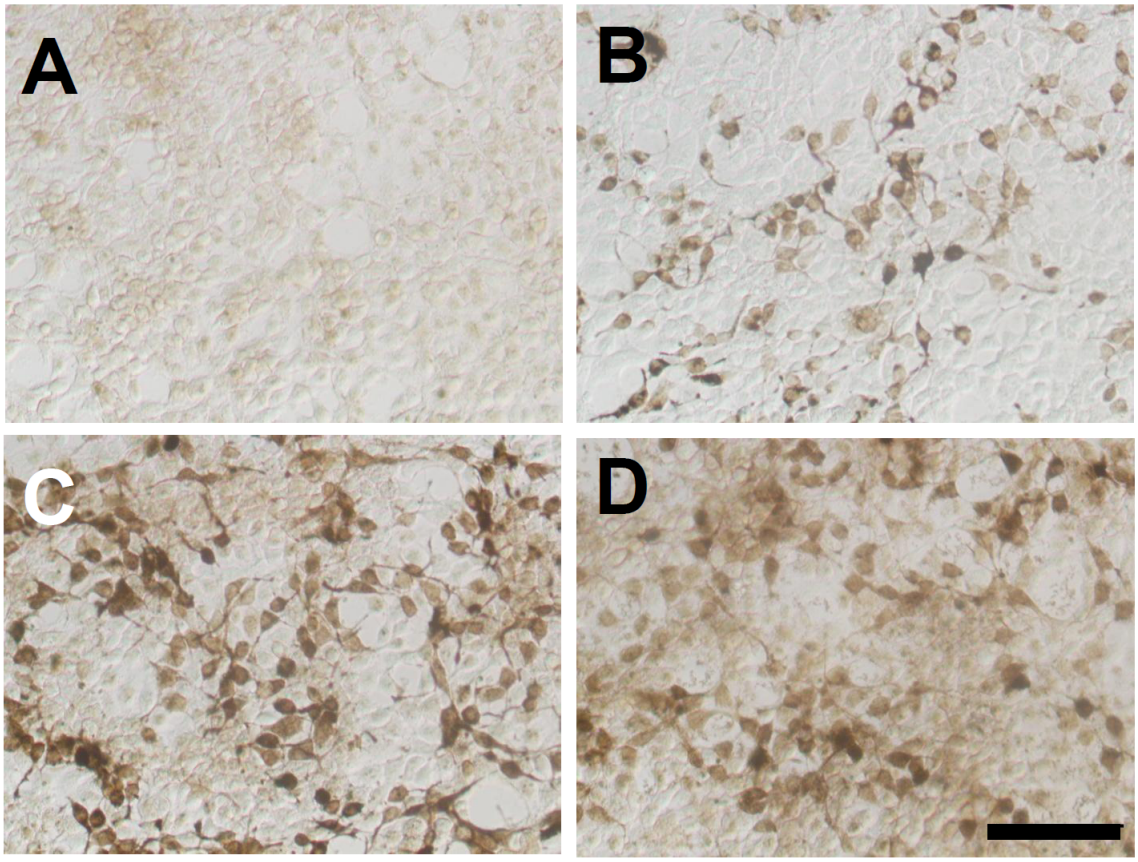

Figure A2.2 Human PAP Dephosphorylates Benfotiamine in a lead histochemistry assay. Cultured HEK 293 cells were transfected either with (A) fEGFP, (B, C) a cherry tagged transmembrane mPAP construct or (D) a secreted mPAP construct. Lead histochemistry was performed at pH 5.6 using (B only) 6 mM TMP as a substrate or (A, C, D) 6 mM Benfotiamine. Cells transfected with mPAP and secreted PAP showed staining patterns that indicate dephosphorylation of the substrate.

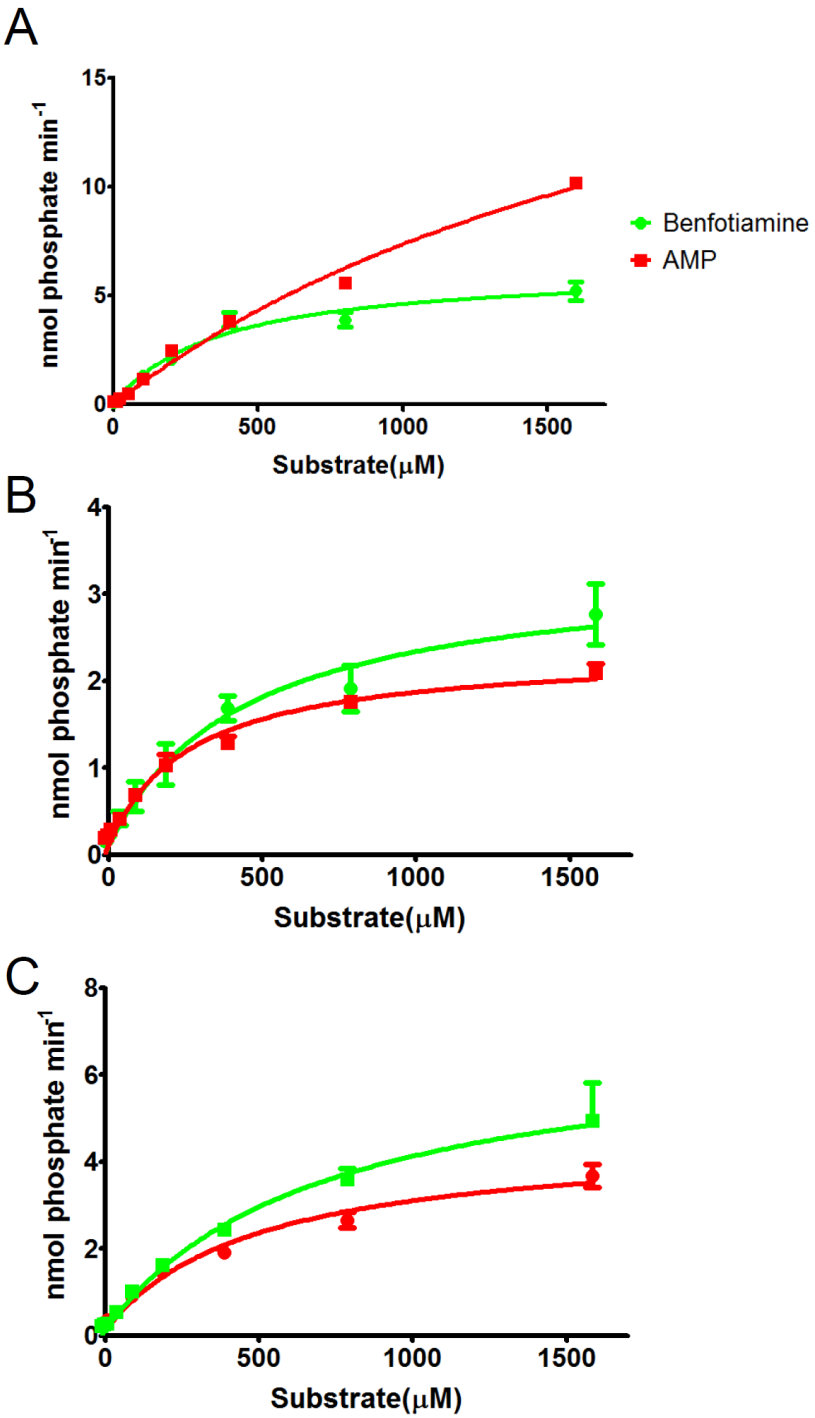

Figure A2.3 Bovine alkaline phosphatase dephosphorylates Benfotiamine.

Plot of initial velocity at the indicated concentrations of AMP or benfotiamine at (A) pH 8.5, (B) pH 7.0 and (C) pH 5.6. K_m values are 731.3 µM and 64.6 µM at pH 8.5,

686.4 µM and 471.4 µM at pH 7.0, and 424.5 µM and 255.7 µM at pH 5.6 for AMP and benfotiamine respectively. 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. GraphPad Prism 5.0 was used to generate curves.

Table A2.1 K_m values (µM) for PAP at different pH values

Substrate	AMP		Benfotiamine			TMP	
pH	**5.6**	**7.0**	**5.6**	**7.0**	**8.5**	**5.6**	**7.0**
PAP	447.9	512.3	101.6	1865	-	354.4	1361
ALP	424.5	686.4	255.7	471.4	64.6	-	-

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