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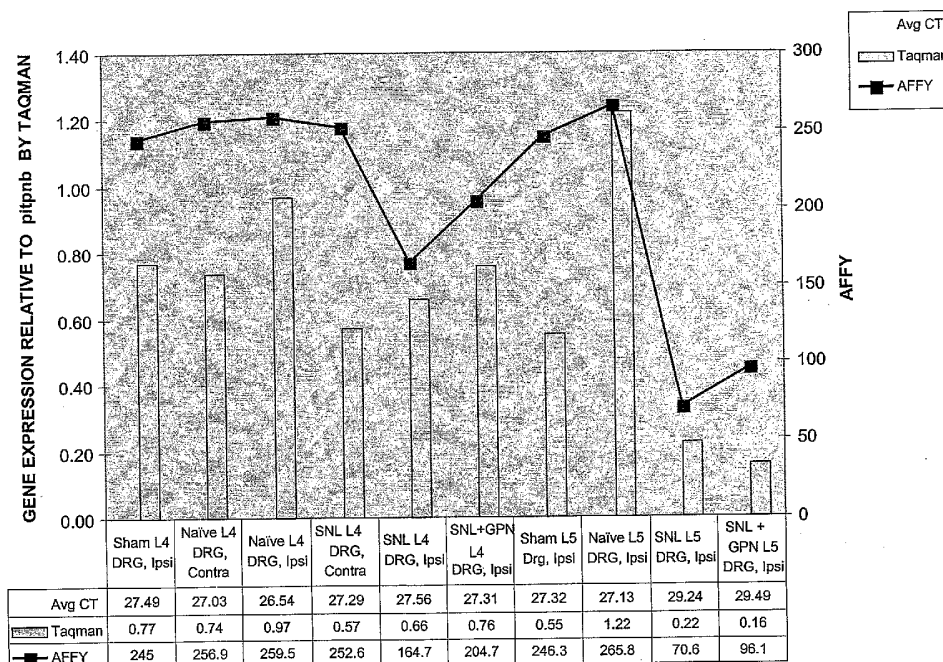
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[Continued on next page]

(54) Title: PAIN-ASSOCIATED GENE PNP5



(57) Abstract: The present invention provides a gene, designated herein as "PNPG5", that is associated with pain. The invention further provides the use of PNP5 gene and its corresponding protein to diagnose a pain state in a cell or tissue and to screen for novel therapeutic compounds useful for treating pain as well as for other indications. The present invention further provides a novel pain-associated molecular complex, comprising PNP5, and at least one other pain-associated transmembrane protein such as PNP2.



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PAIN-ASSOCIATED GENE PNPG5

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[001] The present application claims priority to co-pending U.S. Provisional Application Serial Number 60/562,563 (filed April 14, 2004), entitled "Pain-assoicated Gene PNPG5" and co-pending U.S. Provisional Application Serial Number 60/647,642 (filed January 26, 2005), entitled "Pain-associated Gene PNPG6." The
10 contents of these prior applications are hereby incorporated by reference and in their entirety.

1. FIELD OF THE INVENTION

[002] The present invention provides a gene that is associated with pain and related disorders, in particular, neuropathic pain. This gene, designated herein as
15 the "PNPG5" gene (Purdue Novel Pain Gene 5), and its corresponding protein, can be used in screening methods to identify modulators for the gene or protein as potential therapeutic analgesic candidates and for other indications. The invention also relates to using the PNPG5 gene to generate animal models of pain using transgenic and recombinant technology for screening purposes. The present
20 invention further provides a novel pain-associated molecular complex, which comprises a pain-associated transmembrane protein previously designated as "PNPG2" and PNPG5.

2. BACKGROUND OF THE INVENTION

[003] Pain is the most common symptom for which patients seek medical
25 help, and can be classified as either acute or chronic. Acute pain is precipitated by immediate tissue injury (*e.g.*, a burn or a cut), and is usually self-limited. This form of pain is a natural defense mechanism in response to immediate tissue injury, preventing further use of the injured body part, and withdrawal from the painful stimulus. It is amenable to traditional pain therapeutics, including non-steroidal
30 anti-inflammatory drugs (NSAIDs) and opioids. In contrast, chronic pain is present for an extended period, *e.g.*, for 3 or more months, persisting after an injury has resolved, and can lead to significant changes in a patient's life (*e.g.*, functional

107, Bennett and Plum eds., 20th ed., 1996).

[004] Chronic, debilitating pain represents a significant medical dilemma. In the United States, about 40 million people suffer from chronic recurrent
5 headaches, 35 million people suffer from persistent back pain, 20 million people suffer from osteoarthritis, 2.1 million people suffer from rheumatoid arthritis, and 5 million people suffer from cancer-related pain (Brower, *Nature Biotechnology* 2000; 18: 387-391). Cancer-related pain results from both inflammation and nerve
10 damage. In addition, analgesics are often associated with debilitating side effects such as nausea, dizziness, constipation, respiratory depression and cognitive dysfunction (Brower, *Nature Biotechnology* 2000; 18: 387-391).

[005] Pain can be classified as either “nociceptive” or “neuropathic”, as defined below.

2.1. Nociceptive Pain

15 [006] “Nociceptive pain” is due to activation of pain-sensitive nerve fibers, either somatic or visceral. Nociceptive pain is generally a response to direct tissue damage. The initial trauma typically causes the release of several chemicals including bradykinin, serotonin, substance P, histamine, and prostaglandin. When
20 somatic nerves are involved, the pain is typically experienced as an aching or pressure-like sensation.

[007] Nociceptive pain has traditionally been managed by administering non-opioid analgesics. These analgesics include acetylsalicylic acid, choline magnesium trisalicylate, acetaminophen, ibuprofen, fenoprofen, diflusal, and naproxen, among others. Opioid analgesics, such as morphine, hydromorphone,
25 methadone, levorphanol, fentanyl, oxycodone and oxymorphone, may also be used (Foley, *Pain, In: Cecil Textbook of Medicine*, pp.100-107, Bennett and Plum eds., 20th ed., 1996).

[008] The term "neuropathic pain" refers to pain that is due to injury or disease of the central or peripheral nervous system (McQuay, *Acta Anaesthesiol. Scand.* 1997; 41(1 Pt 2): 175-83; Portenoy, *J. Clin. Oncol.* 1992; 10: 1830-2). In contrast to the immediate pain caused by tissue injury, neuropathic pain can develop days or months after a traumatic injury. Furthermore, while pain caused by tissue injury is usually limited in duration to the period of tissue repair, neuropathic pain frequently is long-lasting or chronic. Moreover, neuropathic pain can occur spontaneously or as a result of stimulation that normally is not painful.

10 [009] Neuropathic pain is associated with chronic sensory disturbances, including spontaneous pain, hyperalgesia (*i.e.*, sensation of more pain than the stimulus would warrant), and allodynia (*i.e.*, a condition in which ordinarily painless stimuli induce the experience of pain). In humans, prevalent symptoms include cold hyperalgesia and mechanical allodynia. Sensitivity to heat is rarely reported. Descriptors that are often used to describe such pain include "lancinating," "burning," or "electric". It is estimated that about 4 million people in North America suffer from chronic neuropathic pain, and of these no more than half achieve adequate pain control (Hansson, *Pain Clinical Updates* 1994; 2(3)).

[010] Examples of neuropathic pain syndromes include those due to disease progression, such as diabetic neuropathy, multiple sclerosis, or post-herpetic neuralgia (shingles); those initiated by injury, such as amputation (phantom-limb pain), or injuries sustained in an accident (*e.g.*, avulsions); and those caused by nerve damage, such as from chronic alcoholism, viral infection, hypothyroidism, uremia, or vitamin deficiencies. Traumatic nerve injuries can also cause the formation of neuromas, in which pain occurs as a result of aberrant nerve regeneration. Stroke (spinal or brain) and spinal cord injury can also induce neuropathic pain. Cancer-related neuropathic pain results from tumor growth compression of adjacent nerves, brain, or spinal cord. In addition, cancer treatments, including chemotherapy and radiation therapy, can also cause nerve injury.

therapies. Treatments for neuropathic pain include opioids, anti-epileptics (*e.g.*, gabapentin, carbamazepine, valproic acid, topiramate, phenytoin), NMDA antagonists (*e.g.*, ketamine, dextromethorphan), topical Lidocaine (for post-herpetic neuralgia), and tricyclic anti-depressants (*e.g.*, fluoxetine (Prozac®), sertraline (Zoloft®), amitriptyline, among others). Neuropathic pain is frequently only partially relieved by high doses of opioids, which are the most commonly used analgesics (Cherny *et al.*, *Neurology* 1994; 44: 857-61.; MacDonald, *Recent Results Cancer Res.* 1991; 121: 24-35.; McQuay, 1997, *supra*). Current therapies may also have serious side effects such as cognitive changes, sedation, and nausea. Many patients suffering from neuropathic pain are elderly or have other medical conditions that limit their tolerance of such side-effects.

2.3. Inflammatory Pain

[012] Chronic somatic pain generally results from inflammatory responses to tissue injury such as nerve entrapment, surgical procedures, cancer or arthritis (Brower, *Nature Biotechnology* 2000; 18: 387-391). Although many types of inflammatory pain are currently treated with NSAIDs, there is much room for improved therapies.

[013] The inflammatory process is a complex series of biochemical and cellular events activated in response to tissue injury or the presence of foreign substances (Levine, *Inflammatory Pain, In: Textbook of Pain*, Wall and Melzack eds., 3rd ed., 1994). Inflammation often occurs at the site of injured tissue, or foreign material, and contributes to the process of tissue repair and healing. The cardinal signs of inflammation include erythema (redness), heat, edema (swelling), pain and loss of function (*ibid.*). The majority of patients with inflammatory pain do not experience pain continually, but rather experience enhanced pain when the inflamed site is moved or touched.

[014] Tissue injury induces the release of inflammatory mediators from damaged cells. These inflammatory mediators include ions (H^+ , K^+), bradykinin, histamine, serotonin (5-HT), ATP and nitric oxide (NO) (Kidd and Urban, *Br. J.*

initiated by activation of the arachidonic acid (AA) pathway. via activation of phospholipase A₂, AA is converted to prostaglandins by cyclooxygenases (Cox-1 and Cox-2), and to leukotrienes by 5-lipoxygenase. The NSAIDs exert their therapeutic action by inhibiting cyclooxygenases. Recruited immune cells release further inflammatory mediators, including cytokines and growth factors, and also activate the complement cascade. Some of these inflammatory mediators (*e.g.*, bradykinin) activate nociceptors directly, leading to spontaneous pain. Others act indirectly via inflammatory cells, stimulating the release of additional pain-inducing (algogenic) agents. Application of inflammatory mediators (*e.g.*, bradykinin, growth factors, prostaglandins) has been shown to produce pain, inflammation and hyperalgesia (increased responsiveness to normally noxious stimuli).

2.4. Genetics

[015] Recent efforts to treat neuropathic pain have focused on identification of genes that are differentially regulated in response to pain stimuli. Using rat models of neuropathic pain, changes in gene and protein expression in the injured part of dorsal root ganglia (DRG) neurons (ipsilateral) compared with the uninjured side (contralateral) or uninjured neurons have been reported (Wang *et al.*, *Neuroscience* 2002; 114: 520-46; Kim *et al.*, *NeuroReport* 2001; 12: 3401-05; Xiao *et al.*, *Proc. Natl. Acad. Sci. USA* 2002; 99: 8361-65; Costigan *et al.*, *BMC Neuroscience* 2002; 3: 16; and Sun *et al.*, *BMC Neuroscience*; 2002; 3: 11). Genes that were found to be up-regulated in injured neurons include those that encode cell-cycle and apoptosis-related proteins; genes associated with neuroinflammation and immune activation, including complement proteins; a gene encoding for calcium channel $\alpha_2\delta$; genes encoding transcription factors; and genes encoding structural proteins or glycoproteins involved in tissue remodeling (Wang *et al.*, *supra*). Genes that were down-regulated compared with uninjured neurons include: neuropeptides such as somatostatin and Substance P; the serotonin 5HT-3 receptor; the glutamate receptor 5 (GluR5); sodium and potassium channels; calcium signaling molecules; and synaptic proteins (Wang *et al.*, *supra*).

injured neurons. Transcription factors determined to be differentially expressed include JunD, NGF1-A and MRG1 (Xiao *et al.*, *supra*; Sun *et al.*, *supra*).

[017] Despite the identification of certain genes that are differentially
5 regulated in models of pain, there remains a need to identify other pain-related genes, and to develop more effective therapies to treat pain, particularly neuropathic pain.

2.5. Nociception and DRG Neurons

[018] Electrophysiological studies have shown the existence of primary
10 sensory neurons that can be excited by noxious heat, intense pressure or irritant chemicals, but not by innocuous stimuli such as warming or light touch (Burgess and Perl, *J. Physiol.* 1967; 190: 541-562). All primary sensory nociceptors make synaptic connections with neurons in the grey matter (dorsal horn) of the spinal cord. Subsets of dorsal horn neurons, in turn, project axons and transmit pain
15 messages to higher brain centers, including the reticular formation, thalamus and ultimately the cerebral cortex.

[019] Fibres that innervate regions of the head and body arise from cell bodies in trigeminal and dorsal root ganglia (DRG), respectively, and can be categorized into three main groups based on anatomical and functional criteria (for
20 review see Julius and Basbaum, *Nature* 2001; 413: 203-210). Cell bodies with the largest diameters give rise to myelinated, rapidly conducting A β primary sensory fibres. Most, but not all, A fibres detect innocuous stimuli applied to skin, muscle and joints and thus do not contribute to pain. By contrast, small- and medium-diameter cell bodies give rise to most of the nociceptors, including unmyelinated,
25 slowly conducting C fibres and thinly myelinated, more rapidly conducting A δ fibres, respectively. It has long been assumed that A and C nociceptors mediate 'first' and 'second' pain, respectively, namely the rapid, acute, sharp pain and the delayed, more diffuse, dull pain evoked by noxious stimuli (Basbaum and Jessell, in

York, 2000).

[020] All sensory systems must convert environmental stimuli into electrochemical signals. However, nociception is unique because individual primary
5 sensory neurons of the 'pain pathway' have the ability to detect a wide range of stimulus modalities, including those of a physical or chemical nature. Compared with sensory neurons of other systems, nociceptors must therefore be equipped with a diverse repertoire of transduction devices. At the same time, markedly different stimuli of a chemical (*e.g.*, capsaicin and acid) or physical (*e.g.*, heat) variety can
10 excite nociceptors by activating a single receptor, enabling the cell to integrate information and respond to complex changes in the physiological environment.

[021] One of the main challenges in the field is to understand how both the specific physiological properties of nociceptors and the "circuits" that they engage in the central nervous system determine pain perception and resultant behavior.
15 Identification of novel molecular markers of nociceptors makes it possible to identify and manipulate the activity of subsets of nociceptors, thereby facilitating the mapping of spinal cord and brainstem circuits that are engaged by specific nociceptor populations. Although opioids and non-steroidal anti-inflammatory agents are current drugs of choice for the treatment of pain, their utility is often
20 limited by unacceptable side-effects due to actions at receptors outside of the pain pathway. Accordingly, identification of novel molecules, receptors, or channels that are expressed in nociceptors should provide new promising targets for the development of more potent and selective anesthetics and analgesics for treating a wide variety of persistent pain conditions.

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2.6. G-Protein Coupled Receptors

[022] G-protein coupled receptors (GPCRs) constitute a major class of proteins responsible for transducing signals within cells (for reviews see Strosberg, *Eur. J. Biochem.* 1991; 196:1-10; Bohm *et al.*, *Biochem J.* 1997; 322: 1-18).
30 GPCRs respond to, among other extracellular signals, calcium ions, hormones,

even photons. Nearly 2000 GPCRs have been reported to date (Nathans and Hogness, *Cell* 1983; 34:807-814; Dixon *et al.*, *Nature* 1986; 321:75-79). GPCRs are classified into over 100 subfamilies according to their homologies, ligand structures, and receptor functions (Ji *et al.*, *J. Biol. Chem.* 1998; 273:17299-17302). GPCRs are characterized by an amino-terminal extracellular domain, a carboxy-terminal intracellular domain, and a serpentine structure that passes through the cell membrane seven times. Hence, such receptors are sometimes also referred to as seven transmembrane (7TM) receptors. These seven transmembrane domains define three extracellular loops and three intracellular loops, in addition to the amino- and carboxy-terminal domains (Ji *et al.*, *J. Biol. Chem.* 1998; 273:17299-17302). Each of the seven TMs is generally composed of 20-27 amino acids. The extracellular portions of GPCRs have a role in recognizing and binding one or more extracellular binding partners (*e.g.*, ligands), whereas the intracellular portions have a role in recognizing and communicating with downstream effector molecules.

[023] When a specific ligand binds to its corresponding GPCR, the ligand typically stimulates the receptor to activate a specific heterotrimeric guanine nucleotide-binding regulatory protein (G protein) that is coupled to the intracellular portion or region of the receptor. The G protein, in turn, transmits a signal to an effector molecule within the cell by either stimulating or inhibiting the activity of that effector molecule. These effector molecules include adenylate cyclase, phospholipases and ion channels. Adenylate cyclase and phospholipases are enzymes that are involved in the production of the second messenger molecules cAMP, inositol triphosphate and diacylglycerol. It is through this sequence of events that an extracellular ligand stimulus exerts intracellular changes through a G protein-coupled receptor. Each such receptor has its own characteristic primary structure, expression pattern, ligand binding profile, and intracellular effector system.

[024] The signal given by GPCRs is terminated rapidly by the activity of regulators of G protein signaling (RGS). In addition, GPCR responsiveness diminishes after repeated or prolonged exposure to the agonist. This process of homologous desensitization of GPCRs (*i.e.*, desensitization via direct action by

coupled receptor kinases (GRKs).

[025] The significance of GPCRs as drug targets lies in their physiological role as mediators between exogenous signals (*i.e.*, ligands) and intracellular responses (see also, Haga and Berstetin eds., *G Protein Coupled Receptors* 1999; CRC Press; Morris and Malbon, *Physiol. Rev.* 1999; 79:1373-1430). GPCRs are useful as drug targets because the binding of natural ligands to GPCR(s) can be moderated by appropriate small molecule drugs so as to modulate intracellular responses (Drews, *Science* 2000; 287:1960-1963; Ma and Zimmel, *Nat. Rev. Drug Discov.* 2002, 1:571-572). GPCRs are associated with almost every major therapeutic category or disease class, including pain, asthma, inflammation, obesity, cancer, as well as cardiovascular, metabolic, gastrointestinal, and CNS diseases (Pierce *et al.*, *Nat. Rev. Mol. Cell Biol.* 2002; 3:639-650). Consequently, GPCRs represent the single most important class of drug targets (Fang *et al.*, *Drug Discovery Today* 2003; 8:755-761). Fifty percent of current drugs target GPCRs and about 20% of the top 50 best-selling drugs are directed to modulating the activities of GPCRs (Drews, *supra* and Ma and Zimmel, *Nat. Rev. Drug Discov.* 2002; 1:571-572).

Several GPCRs, (including metabotropic glutamate receptors (mGluRs)), opioid receptors (including mu, delta, kappa, and ORL-1), cannabinoid receptors, dopamine receptors, serotonin receptors, GABA receptors, muscarinic receptors, adenosine receptors, neurokinin receptors (especially receptors for substance P and NKA), NPY receptors, bradykinin receptors, galanin receptors, and melanocortin-4 receptors) have been shown to be associated with pain and have been used as targets for novel analgesics. The most common GPCRs used as drug targets for pain are opioid receptors (*e.g.*, morphine). Cannabinoids have been used as well. More recently, selective serotonin reuptake inhibitors (SSRIs) have been used to treat migraine headaches.

2.5 The AMIGO Family

[026] Ordered differential display (Matz *et al.*, *Nucleic Acids Res.* 1997, 25: 2541-2542) was used to identify genes in rat neurons that are induced by amphoterin (also known as HMGB1), a protein isolated from perinatal rat brain as a neurite out-growth promoting factor (Kuja-Panula *et al.*, *J. Cell Biol.* 2003, 160: 5 963-973). This technique revealed a differentially-expressed transcript that was cloned and named AMIGO (for amphoterin-induced gene and ORF, GenBank Accession No. AY237729). AMIGO was also found to be induced by the presence of RAGE (receptor for advanced glycation end products and sulphated glycan epitopes) which is a cell surface receptor for amphoterin. The human and mouse 10 homologs of AMIGO (GenBank Accession Nos. AY237007 and AY237008, respectively) were also cloned using data from the rat AMIGO sequence and from EST sequences.

[027] Using a homology search for AMIGO, ESTs were identified and used to clone the rat sequences, AMIGO2 and AMIGO3 (having GenBank Accession 15 Nos. AY237730 and NM_178144, respectively). The AMIGO family (including AMIGO, AMIGO2, and AMIGO3) have the same protein domain organization. At the amino acid level, the similarity between AMIGO and AMIGO2 is 48%, between AMIGO and AMIGO3 is 50%, and between AMIGO2 and AMIGO3 is 48%. The coding sequences for mouse and human AMIGO2 were also determined and 20 assigned GenBank accession numbers AY237006 and AY237005, respectively. Additionally, the coding sequences for mouse and human AMIGO3 were determined and assigned GenBank accession numbers AY237004 and AY237003, respectively.

[028] Kuja-Panula *et al.* (*J. Cell Biol.* 2003, 160:963-973) also show that AMIGO promotes neurite extension of hippocampal neurons and that AMIGO, 25 AMIGO2, and AMIGO3 demonstrate homophillic and heterophillic binding.

[029] Of note, the rat, mouse, and human sequences for AMIGO disclosed by Kuja-Panula *et al.* (*J. Cell Biol.* 2003, 160:963-973) are only for the ORF sequences and do not include untranslated regions (UTRs). The present invention describes a novel function for this gene as a modulator of pain.

[030] A study performed by Rabenau *et al.* (*Onocogene* 2004, 23:5056-5067) showed that AMIGO2 (or DEGA, an acronym for differentially expressed in human gastric adenocarcinomas) mRNA is differentially expressed in human gastric adenocarcinoma cells compared to normal cells. Transfection of cells with an AMIGO2-GFP fusion construct confirmed cell surface localization. Additionally, stable expression of an AMIGO2 antisense construct in a gastric adenocarcinoma cell line led to altered morphology, increased ploidy, chromosomal instability, decreased cell adhesion/migration, and nearly complete abrogation of tumorigenicity in nude mice. Thus, these studies suggest the role of AMIGO2 in gastric adenocarcinoma. However, Rabenau *et al.* does not disclose or suggest the role of AMIGO2 in pain.

3. SUMMARY OF THE INVENTION

[031] The present invention provides genetically modified animals prepared from a mammalian cell that has been genetically modified so that their normal expression of a PNPG5-encoding gene has been changed (*e.g.*, increased, reduced or eliminated), and methods for preparing such cells and animals.

[032] The present invention further provides molecules that can specifically inhibit expression of a PNPG5-encoding nucleic acid molecule or a PNPG5 protein, including without limitation antisense oligonucleotides, ribozymes, triple helix-forming oligonucleotides, and short interfering RNA molecules.

[033] The present invention further provides a method for detecting a pain response in a test cell subjected to a treatment or stimulus or suspected of having been subjected to a treatment or stimulus, said method comprising:

- (a) determining the expression level in the test cell of a nucleic acid molecule encoding a PNPG5 protein; and
- (b) comparing the expression level of the PNPG5-encoding nucleic acid molecule in the test cell to the expression level of

the same nucleic acid molecule in a control cell not subjected to a treatment or stimulus;

wherein a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule in the test cell compared to the expression level of the PNPG5-
5 encoding nucleic acid molecule in the control cell indicates that the test cell is exhibiting a pain response. According to the present invention, the detectable change in the expression level is any statistically significant change and preferably at least a 1.5-fold change as measured by any available technique such as hybridization or quantitative PCR.

10 [034] The present invention further provides a method for detecting a pain response in a test cell, said method comprising:

(a) determining in a test cell capable of expressing a PNPG5 protein the expression level of the protein; and

15 (b) comparing the expression level of the PNPG5 protein in the test cell to the expression level of the PNPG5 protein in a control cell not exhibiting a pain response;

wherein a detectable difference between the expression level of the PNPG5 protein in the test cell and the expression level of the PNPG5 protein in the control cell indicates that the test cell is exhibiting a pain response. According to the present
20 invention, the detectable change in the expression level of a PNPG5 protein is any statistically significant change and preferably at least a 1.5-fold change as measured by any available technique such as protein staining or immunodetection.

[035] The present invention further provides a method for detecting a pain response in a test cell, said method comprising:

25 (a) determining in a test cell capable of expressing a PNPG5 protein the activity level of the PNPG5 protein; and

(b) comparing the activity level of the PNPG5 protein in the test cell to the activity level of the PNPG5 protein in a control cell not exhibiting a pain response;

wherein a detectable difference between the activity level of the PNPG5 protein in the test cell and the activity level of the PNPG5 protein in the control cell indicates that the test cell is exhibiting a pain response. According to the present invention, PNPG5 protein activity can include specific binding of the PNPG5 protein to a binding partner and/or propagation of a transmembrane signal (*e.g.*, stimulation of a G protein such as PNPG2). In a specific embodiment, the activity of the PNPG5 protein is its ability to interact with a protein comprising an LRR domain such as, for example, a PNPG2, PNPG5, AMIGO2, PNPG3/AMIGO3, or truncated PNPG5-TM-minus protein. According to the present invention, the detectable change in the activity level of a PNPG5 protein is any statistically significant change, and preferably at least a 1.5-fold change as measured by any available technique.

[036] As disclosed herein, the test cell can be any cell derived from a tissue of an organism experiencing a feeling of pain or associated disorder. Alternatively, the test cell can be any cell grown *in vitro* under defined conditions. When the test cell is derived from a tissue of an organism experiencing a feeling of pain or associated disorder, the tissue may or may not be known to be located in the region associated with the feeling of pain. The control cell can be any appropriate cell which is known to have not been subjected to any treatment or stimulus associated with pain. Preferably, the control cell is otherwise identical to the test cell. For example, when the test cell is derived from a tissue of an animal experiencing a feeling of pain or associated disorder, the control cell can be derived from an identical tissue or body part of a different animal from, preferably, the same species (or, alternatively, a closely related species) which animal is not experiencing a feeling of pain or associated disorder. Alternatively, the control cell can be derived from an identical tissue or body part of the same animal from which the test cells are derived. However in this latter case, it should be established that the identical tissue or body part has not been subjected to any treatment or stimulus associated

with pain within the timeframe of the experiment. When the test cell is a cell grown *in vitro* under defined conditions, the control cell can be a similar cell grown *in vitro* under identical conditions but without being exposed to a treatment or stimulus inducing a pain state. Additionally, both the test cell and the control cell must have the ability to express a PNP5-encoding polynucleotide molecule or a PNP5 protein. Preferably, the cells used in the method of the invention are neural cells (*e.g.*, dorsal root ganglia (DRG)). In a specific embodiment, the cells are human cells. In another specific embodiment, the cells are derived from an animal model of pain or associated disorder.

10 [037] The present invention further provides a method for identifying a candidate compound useful for modulating the expression of a PNP5-encoding nucleic acid, said method comprising:

15 (a) contacting a first cell with a test compound under conditions sufficient to allow the cell to respond to said contact with the test compound;

(b) determining the expression level of a PNP5-encoding nucleic acid molecule in the cell of step (a); and

20 (c) comparing the expression level of the PNP5-encoding nucleic acid molecule determined in step (b) to the expression level of the PNP5-encoding nucleic acid molecule in a second (control) cell that has not been contacted with the test compound;

wherein a detectable change in the expression level of the PNP5-encoding nucleic acid molecule in the first cell in response to contact with the test compound compared to the expression level of the PNP5-encoding nucleic acid molecule in the second (control) cell that has not been contacted with the test compound indicates that the test compound modulates the expression of the PNP5-encoding nucleic acid and is a candidate compound. In one embodiment of the above method, the test compound is not amphotericin or RAGE (receptor for advanced glycation end products).

[038] Further provided herein is a method for monitoring the efficacy of an analgesic treatment in a cell comprising:

- 5 (a) administering to said cell an analgesic compound under conditions sufficient to allow the cell to respond to said compound;
- (b) determining the expression level of a PNPG5-encoding nucleic acid molecule in the cell prepared in step (a); and
- 10 (c) comparing the expression level of the PNPG5-encoding nucleic acid molecule determined in step (b) to the expression level of the PNPG5-encoding nucleic acid molecule in a second (control) cell that has not been contacted with the analgesic compound;

wherein a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule in the first cell in response to contact with the analgesic compound
15 compared to the expression level of the PNPG5-encoding nucleic acid molecule in the second (control) cell that has not been contacted with the analgesic compound is indicative of the activity of the analgesic compound. In one embodiment of the above method, the test and/or control cells are subjected to a stress corresponding to a painful stimulus.

20 [039] The present invention further provides a method for identifying a candidate compound useful for modulating the expression of a PNPG5 protein, said method comprising:

- 25 (a) contacting a first cell with a test compound under conditions sufficient to allow the cell to respond to said contact with the test compound;
- (b) determining the expression level of a PNPG5 protein in the cell prepared in step (a); and

- (c) comparing the expression level of the PNPG5 protein determined in step (b) to the expression level of the PNPG5 protein in a second (control) cell that has not been contacted with the test compound;

5 wherein a detectable change in the expression level of the PNPG5 protein in the first cell in response to contact with the test compound compared to the expression level of the PNPG5 protein in the second (control) cell that has not been contacted with the test compound, indicates that the test compound modulates the expression of the PNPG5 protein and is a candidate compound.

10 [040] The present invention further provides a method for identifying a candidate compound capable of binding to a PNPG5 protein, said method comprising:

- 15 (a) contacting a PNPG5 protein with a test compound under conditions that permit binding of the test compound to the PNPG5 protein; and
- (b) detecting binding of the test compound to the PNPG5 protein.

[041] An appropriate control is typically included to confirm that the binding of the test compound to the PNPG5 protein is specific. A test compound that can specifically bind to the PNPG5 protein may be considered a candidate
20 compound to be further tested and characterized.

[042] In one embodiment of the above method, the test compound is a protein comprising an LRR domain. Non-limiting examples of test compounds include a PNPG2 protein or a soluble truncated PNPG5-TM-minus protein.

[043] In one embodiment of the above method, the candidate compound is
25 not a PNPG5 protein, AMIGO2 (*e.g.*, rat AMIGO2 with Genbank accession no. AY237730), or PNPG3/AMIGO3 (*e.g.*, rat AMIGO3 with Genbank accession no. NM_178144).

[044] Other examples of PNPG proteins that may be binding partners of PNPG5 can be found in related co-pending U.S. Provisional Patent Application Serial No. 60/584,981 filed on July 2, 2004; related co-pending U.S. Provisional Patent Application Serial No. 60/580,541 filed on June 16, 2004; and related co-
5 pending U.S. Provisional Application Serial Number 60/647,642, filed January 26, 2005.

[045] The present invention further provides a method for identifying a compound useful for modulating an activity of a PNPG5 protein, said method comprising:

- 10 (a) contacting a PNPG5 protein with a test compound under conditions sufficient to allow the PNPG5 protein to respond to said contact with the test compound;
- (b) determining the level of an activity of the PNPG5 protein during or after contact with the test compound; and
- 15 (c) comparing the activity of the PNPG5 protein determined in step (b) to the activity of the PNPG5 protein when it is not in contact with the test compound;

wherein a detectable difference between the activity of the PNPG5 protein in response to contact with the test compound and the activity of the PNPG5 protein
20 when it is not in contact with the test compound indicates that the test compound modulates the activity of the PNPG5 protein. A test compound that can modulate an activity of the PNPG5 protein may be considered a candidate compound to be further tested and characterized.

[046] The present invention further provides a method for identifying a
25 compound capable of modulating binding between a PNPG5 protein and a PNPG2 protein, said method comprising comparing the ability of the PNPG5 protein and the PNPG2 protein to bind together in the presence versus the absence of a test compound.

[047] In one embodiment, the method comprises:

- (a) combining a PNPG5 protein and a PNPG2 protein in the presence of a test compound under conditions that would permit binding of the PNPG5 protein to the PNPG2 protein in the absence of the test compound;
- 5 (b) determining the degree of binding of the PNPG5 protein to the PNPG2 protein in the presence of the test compound; and
- (c) comparing the degree of binding of the PNPG5 protein to the PNPG2 protein in the presence of the test compound with the degree of binding of the PNPG5 protein to the PNPG2 protein in the absence of the test
10 compound;

wherein a detectable difference between the amount of the PNPG5 protein binding to the PNPG2 protein in the presence of the test compound and the amount of the PNPG5 protein binding to the PNPG2 protein in the absence of the test compound indicates that the test compound modulates binding between the PNPG5 protein and
15 the PNPG2 protein.

[048] In one embodiment, the PNPG5 protein and the PNPG2 protein are both soluble proteins. In another embodiment, the PNPG5 protein is a soluble protein and the PNPG2 protein is present in a membrane fraction prepared from a cell. In another embodiment, the PNPG2 protein is a soluble protein and the
20 PNPG5 protein is present in a membrane fraction prepared from a cell. In another embodiment, both the PNPG5 protein and the PNPG2 protein are present in membrane fractions prepared from cells. In another embodiment, the PNPG5 protein is a soluble protein and the PNPG2 protein is present in a membrane of an intact cell. In another embodiment, the PNPG2 protein is a soluble protein and the
25 PNPG5 protein is present in a membrane of an intact cell. In another embodiment, both the PNPG5 protein and the PNPG2 protein are present in membranes of intact cells. In one embodiment, the PNPG5 protein and the PNPG2 protein are present in membranes of different cells. In another embodiment, the PNPG5 protein and the PNPG2 protein are present in the membranes of the same cell. In one embodiment,

the cell or cells in which the protein or proteins are present have been subjected to a stress condition corresponding to a painful stimulus.

[049] In certain embodiments, one or both of the PNPG5 protein and PNPG2 protein are expressed as chimeric/fusion proteins containing an immunodetectable tag or a fluorescent/luminescent reporter polypeptide.

[050] In one embodiment, the amount of the PNPG5 protein bound to the PNPG2 protein is determined by immunoprecipitation or bioluminescence resonance energy transfer (BRET), or mammalian 2-hybrid system, or CytoTrap Two-Hybrid system, or BIACORE methods to detect protein-protein interactions.

[051] In another embodiment, the amount of the PNPG5 protein bound to the PNPG2 protein is determined by measuring a G-protein coupled receptor (GPCR) activity of the PNPG2 protein. According to the present invention, the GPCR activity of PNPG2 can be any GPCR activity known in the art. In a specific embodiment, the GPCR activity of PNPG2 is a change in cAMP levels in the cell. In a specific embodiment, the change in cAMP levels mediated by GPCR activity is measured by the effect on CRE-mediated luciferase activity. In another specific embodiment, the GPCR activity of PNPG2 is a change in calcium levels in the cell. In another specific embodiment, the GPCR activity of PNPG2 is redistribution of an arrestin molecule from the cytoplasm to the plasma membrane.

[052] The present invention further provides a method for treating a condition that can be treated by modulating expression of a PNPG5-encoding nucleic acid molecule or a PNPG5 protein, comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound that modulates expression of a PNPG5-encoding nucleic acid molecule or a PNPG5 protein. Preferably, the treated condition is a pain or pain-related disorder such as, *e.g.*, chronic pain, nociceptive pain, neuropathic pain (including any type of hyperalgesia (*i.e.*, sensation of more pain than the stimulus would warrant) or allodynia (*i.e.*, a condition in which ordinarily painless stimuli induce the experience of pain)), inflammatory or cancer pain, addiction, seizure (including epilepsy),

stroke or ischemia, neurodegenerative disorder (*e.g.*, Parkinson's disease, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), or Huntington's chorea), headache (*e.g.*, general, migraine, cluster or tension), anxiety, depression, asthma, rheumatic disease, osteoarthritis, retinopathy, inflammatory eye disorders, pruritis, ulcer (gastric or duodenal), gastric lesions (*e.g.*, induced by a necrotizing agent), uncontrollable urination (*e.g.*, incontinence), an inflammatory or unstable bladder disorder, inflammatory bowel disease (*e.g.*, Crohn's disease and ulcerative colitis), irritable bowel syndrome (IBS) such as irritable bowel disease (IBD), gastroesophageal reflux disease (GERD), functional dyspepsia (*e.g.*, ulcer-like dyspepsia, dysmotility-like dyspepsia, functional heartburn, or non-ulcer dyspepsia), functional chest pain of presumed oesophageal origin, functional dysphagia, non-cardiac chest pain, symptomatic gastroesophageal disease, gastritis, aerophagia, functional constipation, functional diarrhea, burbulence, chronic functional abdominal pain, recurrent abdominal pain (RAP), functional abdominal bloating, functional biliary pain, functional incontinence, functional ano-rectal pain, chronic pelvic pain, pelvic floor dyssnergia, unspecified functional ano-rectal disorder, cholecystalgia, interstitial cystitis, dysmenorrhea, or dyspareunia.

[053] In a specific embodiment the subject is an animal model of a pain or related disorder. In another specific embodiment, the subject is a mammal such as a human or companion animal (*e.g.*, a dog or cat) suffering from a pain or related disorder.

[054] The present invention further provides a method for treating a condition that can be treated by modulating an activity of a PNPG5 protein, comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound that modulates the activity of a PNPG5 protein.

[055] In a specific embodiment, the activity of the rat PNPG5 protein is its ability to interact with a protein comprising an LRR domain such as, for example, a PNPG2 protein.

[056] The invention further provides a method for treating a condition that can be treated by modulating the degree of binding between a PNPG2 protein and a PNPG5 protein, comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound that modulates the binding between
5 a PNPG2 protein and a PNPG5 protein.

[057] An example of a PNPG2 protein is a rat PNPG2 protein having SEQ ID NO: 38 (see related co-pending U.S. Provisional Patent Application Serial No. 60/580,541 filed on June 16, 2004).

[058] An example of a PNPG5 protein is a rat PNPG5 protein having SEQ
10 ID NO: 2 or a soluble truncated PNPG5-TM-minus protein (e.g., rat PNPG5N-His protein) which lacks the transmembrane (TM) domain of PNPG5 as described in the Examples Section, *infra*.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[059] **Figure 1A** represents analysis of expression of the gene
15 corresponding to EST AA891631 in L4 (unligated) and L5 (ligated) dorsal root ganglia (DRG) in the presence or in the absence of gabapentin (GPN) treatment in a rat sciatic nerve ligation (SNL) model of neuropathic pain. Expression analysis of the gene corresponding to EST AA891631 (SEQ ID NO: 10) in L4 and L5 DRG in naïve (non-operated) and sham-operated animals is also provided. The analysis
20 demonstrates that the expression of the gene corresponding to AA891631 in L5 DRG of SNL animals is down-regulated about 3- to 4-fold as compared to L5 DRG in naïve or sham-operated animals. The analysis of EST AA891631 expression was performed using Affymetrix GeneChip hybridization (line graph; right axis units expressed as gene expression intensity). Apparent down-regulation in gene
25 expression was confirmed by quantitative real time PCR (TaqMan bar graph; left axis units expressed as a relative expression level compared to the control gene, phosphatidylinositol transfer protein, beta (PITPNB)). At the bottom of the graph the data is presented in table format. "Avg CT" refers to the average cycle threshold (CT) value from three replicates in TaqMan analysis. "Taqman" refers to

the relative gene expression level as described in the Examples Section for TaqMan analysis. "AFFY" refers to the gene expression intensity for microchip analysis as described in the Detailed Description and Examples Section.

[060] **Figure 1B** represents analysis of expression of the gene
5 corresponding to EST AA891631 (SEQ ID NO: 10) in 27 naïve rat tissues
demonstrating that EST AA891631 is enriched in DRG. The analysis of EST
AA891631 expression was performed using Affymetrix GeneChip hybridization
(line graph; right axis units expressed as gene expression intensity) and quantitative
real time PCR (TaqMan bar graph; left axis units expressed as relative expression
10 level compared to control gene phosphatidylinositol transfer protein, beta
(PITPNB)).

[061] **Figure 2** represents a Northern blot analysis of total mRNA extracted
from 10 rat tissues using a probe (SEQ ID NO: 8) specific for EST AA891631
(SEQ ID NO: 10). The analysis reveals a rat transcript of ~6 kb in length
15 (indicated by the arrow), which exhibits high expression levels in brain tissue
consistent with Affymetrix and TaqMan analysis of EST AA891631 expression.
Smaller molecular weight bands observed in the Northern likely represent
nonspecific binding. The relative intensity of these smaller bands varies consistently
in a sample dependent fashion as if they vary with the amount of RNA loaded.
20 However, this experiment cannot rule out the existence of additional isoforms of the
gene corresponding to EST AA891631.

[062] **Figure 3** depicts an amino acid sequence alignment of rat PNPG5
(SEQ ID NO: 1), mouse PNPG5 (SEQ ID NO:3), human PNPG5 (SEQ ID NO: 5),
PNPG5 CONTIGab (SEQ ID NO: 32), its mouse ortholog (GenBank Accession No.
25 AK035960, SEQ ID NO: 34), and its human ortholog (GenBank Accession No.
AB032989, SEQ ID NO: 36). The alignment was performed using the AlignX
alignment tool in Vector NTI software (InforMax™, Invitrogen Life Science
Software, Frederick, MD). White, light grey, and dark grey backgrounds indicate
non-similar, identical, and similar amino acids, respectively.

[063] **Figure 4** represents a SMART model (Schultz et al., Proc. Natl. Acad. Sci. USA 1998; 95: 5857-5864 and Letunic et al., Nucleic Acids Res. 2002; 30: 242-244) for the PNPG5 protein which predicts one leucine rich repeat N-terminal domain (LRRNT); two leucine-rich repeats, typical (most populated) subfamily (LRR_TYP); three leucine rich repeats (LRR); one leucine rich repeat C-terminal domain (LRRCT); an immunoglobulin domain (IG); and one transmembrane domain. The leucine-rich repeat is a recently characterized structural motif used in molecular recognition processes as diverse as signal transduction, cell adhesion, cell development, DNA repair and RNA processing. All proteins containing these repeats are thought to be involved in protein-protein interactions. Leucine-rich repeats (LRR) are often flanked by cysteine rich domains, the N-terminal domain (LRRNT) and the C-terminal domain (LRRCT). Detailed descriptions of these types of domains are available on the WorldWideWeb at smart.embl-heidelberg.de/.

15 [064] **Figure 5** represents a brightfield image of naïve rat DRG tissue hybridized *in situ* with ³⁵S-UTP labeled antisense RNA probe (SEQ ID NO: 9) to PNPG5. ³⁵S signal (black dots) shows expression in both small (short arrows) and large neurons (long arrows).

[065] **Figure 6** is a summary of the experimental timeline for surgery, treatment, and behavioral testing of rats used in the SNL model of neuropathic pain.

[066] **Figure 7** is an alignment of PNPG5 fragments by Sequencher (Gene Codes Corporation, Ann Arbor, MI) depicting the relationship between EST AA891631 (referred to as "1631-affy", SEQ ID NO: 10), the rat predicted genomic/cDNA sequence referred to as "Genomic PNPG5" (SEQ ID NO: 22), the orthologous human cDNA sequence (SEQ ID NO:36), the orthologous mouse cDNA sequence (SEQ ID NO: 34), PNPG5 RTPCRa (SEQ ID NO: 30), PNPG5 RTPCRb (SEQ ID NO: 31), and human PNGP5 (SEQ ID NO:5). The box on the mouse ortholog sequence (SEQ ID NO: 34) indicates the location of its open reading frame (ORF).

[067] **Figures 8A and B** represent Western blot analysis of the products of the immunoprecipitation experiments performed in HEK-293 cells transfected with PNPG5-HA containing the rat PNPG5 ORF fused to HA epitope-tag sequence (SEQ ID NO: 39) and/or expression construct pPNPG2-V5 containing the rat PNPG2 ORF (SEQ ID NO: 41) fused to a V5 epitope-tag sequence, as indicated above the loading lanes. Immunoprecipitation was performed using either anti-HA antibodies (A) or anti-V5 antibodies (B) as indicated by “+” in the “IP” row; “-” indicates the whole cell lysate was loaded without an immunoprecipitation step. The Western blot was probed using anti-V5 antibodies (right panel) and anti-HA antibodies (left panel). Co-precipitation of PNPG2 with PNPG5 is detectable in lane 4 in the right panel of Figure 8A and in lane 4 in the left panel of Figure 8B.

[068] **Figures 9A and 9B** represent schematic summary drawings and graphs demonstrating the effect of PNPG5 on the GPCR activity of PNPG2, when expressed in the same cell (A) or in two different cells (B). Expression construct pPNPG5-HA was used for transient transfection of HEK-293 cells. The effect on the GPCR activity of PNPG2 was measured by the effect on cellular cAMP levels. Cellular cAMP levels were monitored by using the pCRE-Luc DNA construct containing a cAMP-responsive CRE promoter element directing the expression of a luciferase gene. In this system, higher levels of cAMP result in higher levels of luciferase gene expression. In Figure 9A, PNPG5 was co-transfected with PNPG2 and pCRE-Luc into HEK-293 cells and then luciferase activity was measured. In Figure 9B, PNPG5 expression construct was individually transfected into HEK-293 cells. The PNPG5-transfected HEK-293 cells were then separately mixed with an equivalent number of HEK-293 cells co-transfected with PNPG2 and pCRE-Luc. Cells (or cell mixtures) expressing only PNPG2 and pCRE-Luc were used as controls. All experiments were performed in triplicate. When present in the same cell (Figure 9A), PNPG5 demonstrates a significant effect on PNPG2 GPCR activity compared to the controls, as evidenced by their ability to significantly reduce luciferase activity (two sample equal variance, two-tailed distribution, t-test P-value of 0.008). When PNPG2 and PNPG5 are present on two different cells (Figure

9B), PNPG5 demonstrates an effect on PNPG2 GPCR activity as evidenced by its ability to reduce luciferase activity (P-values, as above, 0.013).

[069] **Figure 10** is a schematic representation of the full-length PNPG5 (PNPG5-WT), which is a membrane-associated protein containing a transmembrane domain (TM), and a truncated soluble PNPG5-TM-minus polypeptide missing the
5 domain (TM), and a truncated soluble PNPG5-TM-minus polypeptide missing the transmembrane domain and fused to a His tag (PNPG5N-HIS). The PNPG5-TM-minus polypeptide is missing all amino acid sequence from just upstream of the TM domain to the carboxy terminus of the full-length PNPG5 protein.

5. DETAILED DESCRIPTION OF THE INVENTION

10 [070] The present invention provides polynucleotide molecules having nucleotide sequences encoding a protein, designated herein as “PNPG5”. These polynucleotide molecules have been identified as having high sequence similarity to an EST (SEQ ID NO: 10) with GenBank Accession No. AA891631. This EST was isolated among 249 known rat genes and 87 rat expressed-sequence tags (ESTs)
15 identified using microarray technology and validated using quantitative real time PCR, representing genes that are differentially expressed in a rat spinal nerve ligation “SNL” model of neuropathic pain.

[071] The present invention is based on gene expression profiles obtained from a rat spinal nerve ligation “SNL” model of neuropathic pain (Kim and Chung,
20 *Pain* 1992; 50: 355-363) described in the Example section below. This model is created by tightly ligating the L5 and L6 spinal nerves in the rat. The rat L5/6 inter-vertebral disc is innervated by L1 to L6 dorsal root ganglia (DRG). Symptoms induced by this injury include sensitivity to cold and mechanical stimuli, as well as sensitivity to heat. Behaviors indicative of spontaneous pain, such as sudden
25 licking, gentle biting, or pulling of the nails on the operated side, are also observed. These symptoms are attenuated by drugs commonly used in the clinic to treat neuropathic pain, *e.g.*, gabapentin (GPN).

[072] Microarrays containing oligonucleotide probe sets representing around 26,000 unique rat genes (GeneChip®, Rat Genome U34 A, B, and C arrays, Affymetrix, Inc., Santa Clara, CA) were used initially to identify mRNAs which are differentially expressed in SNL rats (with or without gabapentin treatment) as compared to naïve and sham-operated rats. Two-hundred forty nine known genes and eighty seven ESTs were selected on the basis of their differential expression in both injured (L5 and L6) and non-injured (L4) DRGs. To select from among these differentially expressed genes and ESTs, those genes and ESTs likely to be most significant as targets for pain therapeutics, the analysis focused on genes and ESTs the expression of which is co-regulated with the expression of genes known from the prevailing scientific literature to be important as molecular mediators of pain perception and/or restricted in their expression across a panel of 10 normal tissues to the target organ for pain (*i.e.*, the DRG). For the selected set of genes and ESTs, differential expression in the SNL model was validated using quantitative real time PCR (TaqMan®, Applied Biosystems, Foster City, CA) on mRNA samples isolated from an independent pool of animals. The rat EST with GenBank Accession No. AA891631 (herein referred to as EST AA891631) was selected in all of these steps as one of the differentially expressed ESTs, the expression of which is decreased by about 3 to 4-fold in the DRG of rats subjected to the SNL model, and is closely coupled to the expression of multiple known pain genes, such as the valliloid receptor (VR-1), voltage-gated sodium channels (NaN), and SNS/PN3/Nav1.8. A series of BLAST searches identified a partial rat cDNA sequence and human and mouse orthologs corresponding to EST AA891631. The complete rat sequence corresponding to this EST, Genomic PNPG5, was predicted by aligning the mouse cDNA ortholog with the rat genomic sequence corresponding to EST AA891631. Sequencing confirmed the validity of the alignment. Additional BLAST searches for this confirmed rat sequence revealed that this sequence corresponds to the AMIGO coding region (Kuja-Panula *et al.*, *J. Cell Bio.*2003, 160: 963-973).

[073] As further demonstrated herein, the PNPG5 protein can be co-immunoprecipitated in cell lysates with the LRR-containing pain-associated GPCR protein, PNPG2 (*e.g.*, rat PNPG2 protein having SEQ ID NO: 38). Moreover,

PNPG5 can affect the GPCR activity of PNP2, as reflected in their ability to lower cellular cAMP levels. As disclosed in Kuja-Panula *et al.* (*J. Cell Biol.* 2003, 160: 963-973), PNP5, PNP3/AMIGO3, and AMIGO2 demonstrate homophilic and heterophilic binding. Based on these observations, the present invention further provides a novel pain-associated molecular complex comprising a PNP2 protein, a PNP5 protein, and at least one protein selected from the group consisting of PNP5, PNP3/AMIGO3, and AMIGO2.

[074] PNP2 and its association with pain is disclosed in related co-pending U.S. Provisional Patent Application Serial No. 60/580,541 filed on June 16, 2004. This provisional application is incorporated herein by reference in its entirety.

[075] PNP3 and its association with pain is disclosed in related co-pending U.S. Provisional Patent Application Serial No. 60/584,981 filed on July 2, 2004. This provisional application is incorporated herein by reference in its entirety.

5.1. Definitions

[076] As used herein, the term "pain" is art recognized and includes a bodily sensation elicited by noxious chemical, mechanical, or thermal stimuli, in a subject, *e.g.*, a mammal such as a human. The term "pain" includes chronic pain such as lower back pain; pain due to arthritis, *e.g.*, osteoarthritis; joint pain, *e.g.*, knee pain or carpal tunnel syndrome; myofascial pain, and neuropathic pain. The term "pain" further includes acute pain, such as pain associated with muscle strains and sprains; tooth pain; headaches; pain associated with surgery; or pain associated with various forms of tissue injury, *e.g.*, inflammation, infection, and ischemia.

[077] "Neuropathic pain" refers to pain caused by injury or disease of the central or peripheral nervous system. In contrast to the immediate (acute) pain caused by tissue injury, neuropathic pain can develop days or months after a traumatic injury. Neuropathic pain frequently is long-lasting or chronic and is not

limited in duration to the period of tissue repair. Neuropathic pain can occur spontaneously or as a result of stimulation that normally is not painful. Neuropathic pain is sustained by aberrant somatosensory processing, and is associated with chronic sensory disturbances, including spontaneous pain, hyperalgesia (*i.e.*, sensation of more pain than the stimulus would warrant) and allodynia (*i.e.*, a condition in which ordinarily painless stimuli induce the experience of pain). Neuropathic pain includes but is not limited to pain caused by peripheral nerve trauma, viral infection, diabetes mellitus, causalgia, plexus avulsion, neuroma, limb amputation, vasculitis, nerve damage from chronic alcoholism, hypothyroidism, uremia, and vitamin deficiencies, among other causes. Neuropathic pain is one type of pain associated with cancer. Cancer pain can also be “nociceptive” or “mixed.”

[078] “Chronic pain” can be defined as pain lasting longer than three months (Bonica, *Semin. Anesth.* 1986, 5:82-99), and may be characterized by unrelenting persistent pain that is not fully amenable to routine pain control methods. Chronic pain includes, but is not limited to, inflammatory pain, postoperative pain, cancer pain, osteoarthritis pain associated with metastatic cancer, trigeminal neuralgia, acute herpetic and post-herpetic neuralgia, diabetic neuropathy, pain due to arthritis, joint pain, myofascial pain, causalgia, brachial plexus avulsion, occipital neuralgia, reflex sympathetic dystrophy, fibromyalgia, gout, phantom limb pain, burn pain, pain associated with spinal cord injury, multiple sclerosis, reflex sympathetic dystrophy and lower back pain and other forms of neuralgia, neuropathic, and idiopathic pain syndromes.

[079] “Nociceptive pain” is due to activation of pain-sensitive nerve fibers, either somatic or visceral. Nociceptive pain is generally a response to direct tissue damage. The initial trauma causes the release of several chemicals including bradykinin, serotonin, substance P, histamine, and prostaglandin. When somatic nerves are involved, the pain is typically experienced as an aching or pressure-like sensation.

[080] The “dorsal root ganglion” or “DRG” is the cluster of neurons just outside the spinal cord, made of cell bodies of afferent spinal neurons that comprise

the peripheral nervous system (PNS). The cell bodies of sensory nerves that convey somatosensory (sense of touch) information to the brain are found in the DRG. These neurons are unipolar, where the axon splits in two, sending one branch to the sensory receptor and the other to the brain for processing.

5 [081] The term “ipsilateral” refers to the side of the animal on which the L5 and L6 nerves are ligated (wounded) in the rat SNL model of neuropathic pain. The corresponding “ipsilateral” side in a sham-operated animal is the side on which the nerve is exposed but not injured, and the “ipsilateral” side in a naïve animal is the side that would have been injured (*e.g.*, the left side as described in the Section
10 6 (Example), *infra*). The term “contralateral” refers to the unligated (unwounded) side of the animal.

[082] In the context of the present invention insofar as it relates to pain, the terms “treat”, “treatment”, and the like refer to a means to relieve or alleviate the perception of a pain. The terms “treat”, “treatment”, and the like may mean to
15 relieve or alleviate the intensity and/or duration of a pain (*e.g.*, burning sensation, tingling, electric-shock-like feelings, etc.) experienced by a subject in response to a given stimulus (*e.g.*, pressure, tissue injury, cold temperature, etc.). Treatment can occur in a subject (*e.g.*, a human or companion animal) suffering from a pain condition or having one or more symptoms of another condition that can be treated
20 according to the present invention, or in an animal model of pain, such as the SNL rat model of neuropathic pain described herein, or any other animal model of pain. In the context of the present invention insofar as it relates to any of the other conditions recited herein below (other than pain), the terms “treat”, “treatment”, and the like mean to relieve or alleviate at least one symptom associated with such
25 condition, or to slow or reverse the progression of such condition.

[083] An “analgesic” refers to any compound (*e.g.*, small organic molecule, polypeptide, nucleic acid molecule, etc.) that is either known or novel, and useful to treat pain. Specific categories of analgesics include but are not limited to opioids (*e.g.*, morphine, hydromorphone, methadone, levorphanol, fentanyl,
30 oxycodone, oxymorphone), antidepressants (*e.g.*, fluoxetine (Prozac®), sertraline

(Zoloft®), amitriptyline), anti-convulsants (*e.g.*, gabapentin, carbamazepine, valproic acid, topiramate, phenytoin), non-steroidal anti-inflammatory drugs (NSAIDs) and anti-pyretics (such as, *e.g.*, acetaminophen, ibuprofen, fenoprofen, diflusal, naproxen, aspirin and other salicylates (*e.g.*, choline magnesium trisalicylate)), NMDA antagonists (*e.g.*, ketamine, dextromethorphan), and topical Lidocaine (*see also* Sindrup *et al.*, *Pain* 1999; 83: 389-400).

[084] The term "subject" as used herein refers to a mammal (*e.g.*, a rodent such as a mouse or a rat, a pig, a primate, or companion animal (*e.g.*, dog or cat, etc.)). In particular, the term refers to humans.

10 [085] "Expression profile" refers to any description or measurement of one or more of the genes that are expressed by a cell, tissue, or organism under or in response to a particular condition. Expression profiles can identify genes that are up-regulated, down-regulated, or unaffected under particular conditions. Gene expression can be detected at the nucleic acid level or at the protein level. The expression profiling at the nucleic acid level can be accomplished using any available technology to measure gene transcript levels. For example, the method could employ *in situ* hybridization, Northern hybridization or hybridization to a nucleic acid microarray, such as an oligonucleotide microarray, or a cDNA microarray. Alternatively, the method could employ reverse transcriptase-
15 polymerase chain reaction (RT-PCR) such as fluorescent dye-based quantitative real time PCR (TaqMan® PCR). In the Examples section provided below, nucleic acid expression profiles were obtained by (i) hybridization of labeled cRNA derived from total cellular mRNA to Affymetrix GeneChip® oligonucleotide microarrays, (ii) TaqMan® PCR using gene-specific PCR primers, (iii) Northern hybridization, and
20 (iv) *in situ* hybridization. The expression profiling at the protein level can be accomplished using any available technology to measure protein levels, *e.g.*, using peptide-specific capture agent arrays (*see, e.g.*, International PCT Publication No. WO 00/04389).

[086] The term "expressed sequence tag" or "EST" refers to short (usually
30 about 200-600 nt) single-pass sequence reads from one or both ends of a cDNA

clone. Typically, ESTs are produced in large batches by performing a single, automated, sequencing read of cDNA inserts in a cDNA library using a primer based on the vector sequence. As a result, ESTs often correspond to relatively inaccurate (around 2% error) partial cDNA sequences. Since most ESTs are short, they probably will not contain the entire coding region of a large gene (exceeding 200-600 nt in ORF length). Alternatively, or in addition, ESTs may contain non-coding sequences corresponding to untranslated regions of mRNA. ESTs may provide information about the location, expression, and function of the entire gene they represent. They are useful (*e.g.*, as hybridization probes and PCR primers) in identifying full-length genomic and coding sequences as well as in mapping exon-intron boundaries, identifying alternatively spliced transcripts, non-translated transcripts, truly unique genes, and extremely short genes. For a review, *see* Yuan *et al.*, *Pharmacology and Therapeutics* 2001, 91:115-132. In the present application, the term “EST clone” is used to indicate the entire cloned cDNA segment of which only a portion has been initially end-sequenced to produce the “EST” or “EST sequence” that may be stored in public domain sequence databases (*e.g.*, dbEST at NCBI, available on the WorldWideWeb at ncbi.nlm.nih.gov/dbEST/). As with other public domain DNA sequences, these ESTs or EST sequences have accession numbers, and can be analyzed by sequence comparison algorithms such as BLAST, FASTA, DNA Strider, GCG, etc. The Affymetrix GeneChip arrays used in the Examples section below include probesets (consisting of 25 nt oligonucleotides) designed to measure mRNA levels of the gene encompassing the EST and are annotated by Affymetrix with the accession number for the relevant EST sequence. Herein, such probesets are referred to by their EST accession number (*e.g.*, Accession No. AA891631 for EST corresponding to rat PNPG5).

[087] The terms “array” and “microarray” are used interchangeably and refer generally to any ordered arrangement (*e.g.*, on a surface or substrate) of different molecules, referred to herein as “probes.” Each different probe of an array is capable of specifically recognizing and/or binding to a particular molecule, which is referred to herein as its “target,” in the context of arrays. Examples of

typical target molecules that can be detected using microarrays include mRNA transcripts, cDNA molecules, cRNA molecules, and proteins. As disclosed in the Examples section below, at least one target detectable by the Affymetrix GeneChip® microarray used as described herein is a PNPG5-encoding nucleic acid (such as an mRNA transcript, or a corresponding cDNA or cRNA molecule).

[088] Microarrays are useful for simultaneously detecting the presence, absence and quantity of a plurality of different target molecules in a sample (such as an mRNA preparation isolated from a relevant cell, tissue, or organism, or a corresponding cDNA or cRNA preparation). The presence and quantity, or absence, of a probe's target molecule in a sample may be readily determined by analyzing whether (and how much of) a target has bound to a probe at a particular location on the surface or substrate.

[089] In a preferred embodiment, arrays used in the present invention are "addressable arrays" where each different probe is associated with a particular "address". For example, in a preferred embodiment where the probes are immobilized on a surface or a substrate, each different probe of the addressable array is immobilized at a particular, known location on the surface or substrate. The presence or absence of that probe's target molecule in a sample may therefore readily be determined by simply detecting whether a target has bound to that particular location on the surface or substrate.

[090] The arrays according to the present invention are preferably nucleic acid arrays (also referred to herein as "transcript arrays" or "hybridization arrays") that comprise a plurality of nucleic acid probes immobilized on a surface or substrate. The different nucleic acid probes are complementary to, and therefore can hybridize to, different target nucleic acid molecules in a sample. Thus, such probes can be used to simultaneously detect the presence and quantity of a plurality of different nucleic acid molecules in a sample, to determine the expression level of a plurality of different genes, *e.g.*, the presence and abundance of different mRNA molecules, or of nucleic acid molecules derived therefrom (for example, cDNA or cRNA).

[091] There are two major types of microarray technology; spotted cDNA arrays and manufactured oligonucleotide arrays. The Examples section below describes the use of high density oligonucleotide Affymetrix GeneChip® arrays.

[092] The arrays are preferably reproducible, allowing multiple copies of a given array to be produced and the results from each easily compared to each other. Preferably the microarrays are small, usually smaller than 5 cm², and are made from materials that are stable under binding (*e.g.*, nucleic acid hybridization) conditions. A given binding site or unique set of binding sites in the microarray will specifically bind the target (*e.g.*, the mRNA of a single gene in the cell). Although there may be more than one physical binding site (hereinafter “site”) per specific target, for the sake of clarity the discussion below will assume that there is a single site. It will be appreciated that when cDNA complementary to the RNA of a cell is made and hybridized to a microarray under suitable hybridization conditions, the level or degree of hybridization to the site in the array corresponding to any particular gene will reflect the prevalence in the cell of mRNA transcribed from that gene. For example, when detectably labeled (*e.g.*, with a fluorophore) cDNA complementary to the total cellular mRNA is hybridized to a microarray, any site on the array corresponding to a gene (*i.e.*, capable of specifically binding a nucleic acid product of the gene) that is not transcribed in the cell will have little or no signal, while a gene for which the encoded mRNA is highly prevalent will have a relatively strong signal.

[093] By way of example, GeneChip expression analysis (Affymetrix, Santa Clara, CA) generates data for the assessment of gene expression profiles and other biological assays. Oligonucleotide expression arrays simultaneously and quantitatively “interrogate” thousands of mRNA transcripts (genes or ESTs), simplifying large genomic studies. Each transcript can be represented on a probe array by multiple probe pairs to differentiate among closely related members of gene families. Each probe set contains millions of copies of a specific oligonucleotide probe, permitting the accurate and sensitive detection of even low-intensity mRNA hybridization patterns. After hybridization intensity data is captured, *e.g.*, using optical detection systems (*e.g.*, a scanner), software can be used to automatically

calculate intensity values for each probe cell. Probe cell intensities can be used to calculate an average intensity for each gene, which correlates with mRNA abundance levels. Expression data can be quickly sorted based on any analysis parameter and displayed in a variety of graphical formats for any selected subset of
5 genes. Gene expression detection technologies include, among others, the research products manufactured and sold by Hewlett-Packard, Perkin-Elmer and Gene Logic.

[094] An "antisense" nucleic acid molecule or oligonucleotide is a single stranded nucleic acid molecule, which may be DNA, RNA, a DNA-RNA chimera, or a derivative thereof, which, upon hybridizing under physiological conditions with
10 complementary bases in an RNA or DNA molecule of interest, inhibits the expression of the corresponding gene by inhibiting, *e.g.*, mRNA transcription, mRNA splicing, mRNA transport, or mRNA translation or by decreasing mRNA stability. As presently used, "antisense" broadly includes RNA-RNA interactions, RNA-DNA interactions, and RNase-H mediated arrest. Antisense nucleic acid
15 molecules can be encoded by a recombinant gene for expression in a cell (see, *e.g.*, U.S. Patents No. 5,814,500 and 5,811,234), or alternatively they can be prepared synthetically (see, *e.g.*, U.S. Patent No. 5,780,607). According to the present invention, the involvement of PNPG5 in regulation of any particular pain condition may be identified, modulated and studied using antisense nucleic acids derived on
20 the basis of PNPG5-encoding nucleic acid molecules of the invention.

[095] The term "ribozyme" is used to refer to a catalytic RNA molecule capable of cleaving RNA substrates. Ribozyme specificity is dependent on complementary RNA-RNA interactions (for a review, see Cech and Bass, *Annu. Rev. Biochem.* 1986; 55: 599-629). Two types of ribozymes, hammerhead and
25 hairpin, have been described. Each has a structurally distinct catalytic center. The present invention contemplates the use of ribozymes designed on the basis of the PNPG5-encoding nucleic acid molecules of the invention to induce catalytic cleavage of the corresponding mRNA, thereby inhibiting expression of the PNPG5 gene. Ribozyme technology is described further in *Intracellular Ribozyme*
30 *Applications: Principals and Protocols*, Rossi and Couture ed., Horizon Scientific Press, 1999.

[096] The term “RNA interference” or “RNAi” refers to the ability of double stranded RNA (dsRNA) to suppress the expression of a specific gene of interest in a homology-dependent manner. It is currently believed that RNA interference acts post-transcriptionally by targeting mRNA molecules for degradation. RNA interference commonly involves the use of dsRNAs that are greater than 500 bp; however, it can also be mediated through small interfering RNAs (siRNAs) or small hairpin RNAs (shRNAs), which can be 10 or more nucleotides in length and are typically 18 or more nucleotides in length. For reviews, see Bosner and Labouesse, *Nature Cell Biol.* 2000; 2: E31-E36 and Sharp and Zamore, *Science* 2000; 287: 2431-2433.

[097] The term “nucleic acid hybridization” refers to anti-parallel hydrogen bonding between two single-stranded nucleic acids, in which A pairs with T (or U if an RNA nucleic acid) and C pairs with G. Nucleic acid molecules are “hybridizable” to each other when at least one strand of one nucleic acid molecule can form hydrogen bonds with the complementary bases of another nucleic acid molecule under defined stringency conditions. Stringency of hybridization is determined, *e.g.*, by (i) the temperature at which hybridization and/or washing is performed, and (ii) the ionic strength and (iii) concentration of denaturants such as formamide of the hybridization and washing solutions, as well as other parameters. Hybridization requires that the two strands contain substantially complementary sequences. Depending on the stringency of hybridization, however, some degree of mismatches may be tolerated. Under “low stringency” conditions, a greater percentage of mismatches are tolerable (*i.e.*, will not prevent formation of an anti-parallel hybrid). See *Molecular Biology of the Cell*, Alberts *et al.*, 3rd ed., New York and London: Garland Publ., 1994, Ch. 7.

[098] Typically, hybridization of two strands at high stringency requires that the sequences exhibit a high degree of complementarity over an extended portion of their length. Examples of high stringency conditions include: hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% SDS, 1 mM EDTA at 65°C, followed by washing in 0.1x SSC/0.1% SDS (where 1x SSC is 0.15 M NaCl, 0.15 M Na citrate) at 68°C or for oligonucleotide molecules washing in

6xSSC/0.5% sodium pyrophosphate at about 37°C (for 14 nucleotide-long oligos), at about 48°C (for about 17 nucleotide-long oligos), at about 55°C (for 20 nucleotide-long oligos), and at about 60°C (for 23 nucleotide-long oligos)).

[009] Conditions of intermediate or moderate stringency (such as, for
5 example, an aqueous solution of 2xSSC at 65°C; alternatively, for example, hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C) and low stringency (such as, for example, an aqueous solution of 2xSSC at 55°C), require correspondingly less overall complementarity for hybridization to occur between two sequences. Specific
10 temperature and salt conditions for any given stringency hybridization reaction depend on the concentration of the target DNA and length and base composition of the probe, and are normally determined empirically in preliminary experiments, which are routine (see Southern, *J. Mol. Biol.* 1975; 98: 503; Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 2, ch. 9.50, CSH
15 Laboratory Press, 1989; Ausubel *et al.* (eds.), 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3).

[0100] As used herein, the term “standard hybridization conditions” refers to hybridization conditions that allow hybridization of two nucleotide molecules having
20 at least 75% sequence identity. According to a specific embodiment, hybridization conditions of higher stringency may be used to allow hybridization of only sequences having at least 80% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity.

[0101] Nucleic acid molecules that “hybridize” to any of the PNPG5-
25 encoding nucleic acids of the present invention may be of any length. In one embodiment, such nucleic acid molecules are at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, and at least 70 nucleotides in length. In another embodiment, nucleic acid molecules that hybridize are of about the same length as the particular PNPG5-encoding nucleic acid.

[0102] The term "homologous" as used in the art commonly refers to the relationship between nucleic acid molecules or proteins that possess a "common evolutionary origin," including nucleic acid molecules or proteins within superfamilies (*e.g.*, the immunoglobulin superfamily) and nucleic acid molecules or proteins from different species (Reeck *et al.*, *Cell* 1987; 50: 667). Such nucleic acid molecules or proteins have sequence homology, as reflected by their sequence similarity, whether in terms of substantial percent similarity or the presence of specific residues or motifs at conserved positions.

[0103] The terms "percent (%) sequence similarity", "percent (%) sequence identity", and the like, generally refer to the degree of identity or correspondence between different nucleotide sequences of nucleic acid molecules or amino acid sequences of proteins that may or may not share a common evolutionary origin (see Reeck *et al.*, *supra*). Sequence identity can be determined using any of a number of publicly available sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin), etc.

[0104] To determine the percent identity between two amino acid sequences or two nucleic acid molecules, the sequences are aligned for optimal comparison purposes. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, percent identity = number of identical positions/total number of positions (*e.g.*, overlapping positions) x 100). In one embodiment, the two sequences are, or are about, of the same length. The percent identity between two sequences can be determined using techniques similar to those described below, with or without allowing gaps. In calculating percent sequence identity, typically exact matches are counted.

[0105] The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 1990, 87:2264, modified as in Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 1993, 90:5873-

5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, *J. Mol. Biol.* 1990; 215: 403. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to sequences of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to protein sequences of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, *Nucleic Acids Res.* 1997, 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationship between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See ncbi.nlm.nih.gov/BLAST/ on the WorldWideWeb. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, *CABIOS* 1988; 4: 11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

[0106] In a preferred embodiment, the percent identity between two amino acid sequences is determined using the algorithm of Needleman and Wunsch (*J. Mol. Biol.* 1970, 48:444-453), which has been incorporated into the GAP program in the GCG software package (Accelrys, Burlington, MA; available at accelrys.com on the WorldWideWeb) using either a Blossum 62 matrix or a PAM250 matrix, a gap weight of 16, 14, 12, 10, 8, 6, or 4, and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package using a NWSgapdna.CMP matrix, a gap weight of 40, 50, 60, 70, or 80, and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and one that can be used if the practitioner is uncertain about what parameters should be applied to determine if a molecule is a sequence identity or homology limitation of

the invention) is using a Blossum 62 scoring matrix with a gap open penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[0107] In addition to the cDNA sequences encoding rat, mouse and human PNPG5 proteins (as shown in SEQ ID NOS: 1, 3 and 5, respectively), the present invention further provides polynucleotide molecules comprising nucleotide sequences having certain percentage sequence identities to any of the aforementioned sequences. Such sequences preferably hybridize under conditions of moderate or high stringency as described above, and may include species orthologs.

[0108] As used herein, the term "orthologs" refers to genes in different species that apparently evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function through the course of evolution. Identification of orthologs can provide reliable prediction of gene function in newly sequenced genomes. Sequence comparison algorithms that can be used to identify orthologs include without limitation BLAST, FASTA, DNA Strider, and the GCG pileup program. Orthologs often have high sequence similarity, as can be seen in the case of rat, mouse and human PNPG5 proteins of the present invention. For example, as shown in **Figure 3** using AlignX Vector NTI algorithm (InforMax™, Invitrogen Life Science Software, Frederick, MD), the rat PNPG5 protein sequence is 97% identical to the mouse PNPG5 protein sequence and the rat PNPG5 protein sequence is 89% identical to the human PNPG5 protein sequence.

[0109] The present invention encompasses all orthologs of PNPG5. In addition to rat, mouse and human orthologs, particularly useful PNPG5 orthologs of the present invention are monkey, porcine, canine (dog), and guinea pig orthologs.

[0110] As used herein, the term "isolated" means that the material being referred to has been removed from the environment in which it is naturally found, and is characterized to a sufficient degree to establish that it is present in a particular sample. Such characterization can be achieved by any standard technique, such as, *e.g.*, sequencing, hybridization, immunoassay, functional assay, expression, size determination, or the like. Thus, a biological material can be "isolated" if it is free

of cellular components, *i.e.*, components of the cells in which the material is found or produced in nature. For nucleic acid molecules, an isolated nucleic acid molecule or isolated polynucleotide molecule, or an isolated oligonucleotide, can be a PCR product, an mRNA transcript, a cDNA molecule, or a restriction fragment.

5 A nucleic acid molecule excised from the chromosome that it is naturally a part of is considered to be isolated. Such a nucleic acid molecule may or may not remain joined to regulatory, or non-regulatory, or non-coding regions, or to other regions located upstream or downstream of the gene when found in the chromosome. Nucleic acid molecules that have been spliced into vectors such as plasmids, 10 cosmids, artificial chromosomes, phages and the like are considered isolated. In a particular embodiment, a PNPG5-encoding nucleic acid spliced into a recombinant vector, and/or transformed into a host cell, is considered to be "isolated".

[0111] Isolated nucleic acid molecules and isolated polynucleotide molecules of the present invention do not encompass uncharacterized clones in man-made 15 genomic or cDNA libraries.

[0112] A protein that is associated with other proteins and/or nucleic acids with which it is associated in an intact cell, or with cellular membranes if it is a membrane-associated protein, is considered isolated if it has otherwise been removed from the environment in which it is naturally found and is characterized to 20 a sufficient degree to establish that it is present in a particular sample. A protein expressed from a recombinant vector in a host cell, particularly in a cell in which the protein is not naturally expressed, is also regarded as isolated.

[0113] An isolated organelle, cell, or tissue is one that has been removed from the anatomical site (cell, tissue or organism) in which it is found in the source 25 organism.

[0114] An isolated material may or may not be "purified". The term "purified" as used herein refers to a material (*e.g.*, a nucleic acid molecule or a protein) that has been isolated under conditions that detectably reduce or eliminate the presence of other contaminating materials. Contaminants may or may not

include native materials from which the purified material has been obtained. A purified material preferably contains less than about 90%, less than about 75%, less than about 50%, less than about 25%, less than about 10%, less than about 5%, or less than about 2% by weight of other components with which it was originally associated.

[0115] Methods for purification are well-known in the art. For example, nucleic acids or polynucleotide molecules can be purified by precipitation, chromatography (including preparative solid phase chromatography, oligonucleotide hybridization, and triple helix chromatography), ultracentrifugation, and other means. Polypeptides can be purified by various methods including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reverse-phase HPLC, gel filtration, affinity chromatography, ion exchange and partition chromatography, precipitation and salting-out chromatography, extraction, and counter-current distribution. Cells can be purified by various techniques, including centrifugation, matrix separation (*e.g.*, nylon wool separation), panning and other immunoselection techniques, depletion (*e.g.*, complement depletion of contaminating cells), and cell sorting (*e.g.*, fluorescence activated cell sorting (FACS)). Other purification methods are possible. The term “substantially pure” indicates the highest degree of purity that can be achieved using conventional purification techniques currently known in the art. In the context of analytical testing of the material, “substantially free” means that contaminants, if present, are below the limits of detection using current techniques, or are detected at levels that are low enough to be acceptable for use in the relevant art, for example, no more than about 2-5% (w/w). Accordingly, with respect to the purified material, the term “substantially pure” or “substantially free” means that the purified material being referred to is present in a composition where it represents 95% (w/w) or more of the weight of that composition. Purity can be evaluated by chromatography, gel electrophoresis, immunoassay, composition analysis, biological assay, or any other appropriate method known in the art.

[0116] The term “about” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend

in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within an acceptable standard deviation, per the practice in the art. Alternatively, “about” can mean a range of up to $\pm 20\%$, preferably up to $\pm 10\%$, more preferably up to $\pm 5\%$, and
5 more preferably still up to $\pm 1\%$ of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated, the term “about” is
10 implicit and in this context means within an acceptable error range for the particular value.

[0117] The term “degenerate variants” of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position.

[0118] The term “modulator” refers to a compound that differentially affects
15 the expression or activity of a gene or gene product (*e.g.*, nucleic acid molecule or protein), for example, in response to a stimulus that normally activates or represses the expression or activity of that gene or gene product when compared to the expression or activity of the gene or gene product not contacted with the stimulus. In one embodiment, the gene or gene product the expression or activity of which is
20 being modulated includes a gene, cDNA molecule or mRNA transcript that encodes a mammalian PNPG5 protein such as, *e.g.*, a rat, mouse, companion animal, or human PNPG5 protein. Examples of modulators of the PNPG5-encoding nucleic acids of the present invention include without limitation antisense nucleic acids, ribozymes, and RNAi oligonucleotides.

25 [0119] A “test compound” is a molecule that can be tested for its ability to act as a modulator of a gene or gene product. Test compounds can be selected without limitation from small inorganic and organic molecules (*i.e.*, those molecules of less than about 2 kD, and more preferably less than about 1 kD in molecular weight), polypeptides (including native ligands, antibodies, antibody fragments, and
30 other immunospecific molecules), oligonucleotides, polynucleotide molecules, and

derivatives thereof. In various embodiments of the present invention, a test compound is tested for its ability to modulate the expression of a mammalian PNPG5-encoding nucleic acid or PNPG5 protein or to bind to a mammalian PNPG5 protein. A compound that modulates a nucleic acid or protein of interest is
5 designated herein as a “candidate compound” or “lead compound” suitable for further testing and development. Candidate compounds include, but are not necessarily limited to, the functional categories of agonist and antagonist.

[0120] An “agonist” is defined herein as a compound that interacts with (*e.g.*, binds to) a nucleic acid molecule or protein, and promotes, enhances,
10 stimulates or potentiates the biological expression or function of the nucleic acid molecule or protein. The term “partial agonist” is used to refer to an agonist which interacts with a nucleic acid molecule or protein, but promotes only partial function of the nucleic acid molecule or protein. A partial agonist may also inhibit certain functions of the nucleic acid molecule or protein with which it interacts. An
15 “antagonist” interacts with (*e.g.*, binds to) and inhibits or reduces the biological expression or function of the nucleic acid molecule or protein. An “inverse agonist” is a compound that interacts with a nucleic acid molecule or protein and elicits an effect opposite to that of an agonist.

[0121] The phrase “similar or identical expression” (and the like) as used
20 herein refers to an expression level of a PNPG5 gene or gene product (*i.e.*, an mRNA transcript or protein) in a first cell that is $\pm 30\%$, preferably $\pm 20\%$, and more preferably $\pm 10\%$ of a given numerical value of the expression level of the same PNPG5 gene or gene product in a second comparator (or control) cell as determined by any quantitative assay known in the art. Preferably, the second cell
25 is either (i) the same type of cell, and preferably from the same type of tissue, as the first cell, or (ii) is from the same cell line as the first cell. This second cell is also referred to herein as a “control cell” or “corresponding cell”. The first and second cells are preferably, but need not be, otherwise incubated and treated under the same conditions.

[0122] The phrase “activity which is the same as or similar to” (and the like) as used herein refers to at least one activity of a protein that is within $\pm 90\%$, preferably within $\pm 50\%$, and more preferably within $\pm 10\%$ of a given numerical value of at least one activity of another protein as determined by any quantitative assay known in the art. More narrowly, this phrase is used herein to refer to at least one activity of a protein that is within $\pm 90\%$, preferably within $\pm 50\%$, and more preferably within $\pm 10\%$ of a given numerical value of at least one activity of the full-length PNPG5 protein having the amino acid sequence of SEQ ID NO: 2, 4 or 6. According to the present invention, the activity of a PNPG5 protein can be any activity currently known in the art or identified later. In a specific embodiment, the activity of the PNPG5 protein is its ability to interact with (e.g., bind to and/or co-immunoprecipitate with) a protein comprising a Leucine Rich Repeat (LRR) domain such as, for example, a PNPG2, PNPG5, PNPG3/AMIGO3, or AMIGO2 protein. In another specific embodiment, the activity of the PNPG5 protein is the stimulation of neurite extension of hippocampal neurons (Kuja-Panula *et al.*, *J. Cell Biol.*, 160:963-973 (2003)). In yet another specific embodiment, the activity of the PNPG5 protein is the ability to promote PNPG2-mediated signaling. The term “biologically active fragment” refers to a peptide fragment of a protein, which fragment has at least one activity constituting $\pm 90\%$, preferably $\pm 50\%$, and more preferably $\pm 10\%$ of a given numerical value of at least one activity of the corresponding full-length protein, as determined by any quantitative assay known in the art.

[0123] The term “detectable change” as used herein in relation to an expression level of a gene or gene product (e.g., PNPG5) or activity of a gene product means any statistically significant change and preferably at least a 1.5-fold change as measured by any available technique such as hybridization or quantitative PCR.

[0124] As used herein, the term “specific binding” refers to the ability of one molecule, typically an antibody, polynucleotide, polypeptide, or a small molecule ligand to contact and associate with another specific molecule, even in the presence of many other diverse molecules. “Immunospecific binding” refers to the

ability of an antibody to specifically bind to (or to be “specifically immunoreactive with”) its corresponding antigen.

[0125] “Amplification” of DNA as used herein denotes the use of exponential amplification techniques known in the art such as the polymerase chain reaction (PCR), and non-exponential amplification techniques such as linked linear amplification, that can be used to increase the concentration of a particular DNA sequence present in a mixture of DNA sequences. For a description of PCR, see Saiki *et al.*, *Science* 1988, 239:487 and U.S. Patent No. 4,683,202. For a description of linked linear amplification, see U.S. Patent Nos. 6,335,184 and 6,027,923; Reyes *et al.*, *Clinical Chemistry* 2001; 47: 131-40; and Wu *et al.*, *Genomics* 1989; 4: 560-569.

[0126] As used herein, the phrase “sequence-specific oligonucleotides” refers to oligonucleotides that can be used to detect the presence of a specific nucleic acid molecule, or that can be used to amplify a particular segment of a specific nucleic acid molecule for which a template is present. Such oligonucleotides are also referred to as “primers” or “probes.” In a specific embodiment, “probe” is also used to refer to an oligonucleotide, for example about 25 nucleotides in length, attached to a solid support for use on “arrays” and “microarrays” described below.

[0127] The term “host cell” refers to any cell of any organism that is selected, modified, transformed, grown, used or manipulated in any way so as, *e.g.*, to clone a recombinant vector that has been transformed into that cell, or to express a recombinant protein such as, *e.g.*, a PNPG5 protein of the present invention. Host cells are useful in screening and other assays, as described below.

[0128] As used herein, the terms “transfected cell” and “transformed cell” both refer to a host cell that has been genetically modified to express or over-express a nucleic acid encoding a specific gene product of interest such as, *e.g.*, a PNPG5 protein or a fragment thereof. Any eukaryotic or prokaryotic cell can be used, although eukaryotic cells are preferred, vertebrate cells are more preferred, and mammalian cells are the most preferred. In the case of multi-subunit ion

channels, nucleic acids encoding the several subunits are preferably co-expressed by the transfected or transformed cell to form a functional channel. Transfected or transformed cells are suitable to conduct an assay to screen for compounds that modulate the function of the gene product. A typical "assay method" of the present invention makes use of one or more such cells, *e.g.*, in a microwell plate or some other culture system, to screen for such compounds. The effects of a test compound can be determined on a single cell, or on a membrane fraction prepared from one or more cells, or on a collection of intact cells sufficient to allow measurement of activity.

10 [0129] The term "recombinantly engineered cell" refers to any prokaryotic or eukaryotic cell that has been genetically manipulated to express or over-express a nucleic acid of interest, *e.g.*, a PNPG5-encoding nucleic acid of the present invention, by any appropriate method, including transfection, transformation or transduction. The term "recombinantly engineered cell" also refers to a cell that
15 has been engineered to activate an endogenous nucleic acid, *e.g.*, the endogenous PNPG5-encoding gene in a rat, mouse or human cell, which cell would not normally express that gene product or would express the gene product at only a sub-optimal level.

[0130] The terms "vector", "cloning vector" and "expression vector" refer
20 to recombinant constructs including, *e.g.*, plasmids, cosmids, phages, viruses, and the like, with which a nucleic acid molecule (*e.g.*, a PNPG5-encoding nucleic acid or PNPG5 siRNA-expressing nucleic acid) can be introduced into a host cell so as to, *e.g.*, clone the vector or express the introduced nucleic acid molecule. Vectors may further comprise selectable markers.

25 [0131] The terms "mutant", "mutated", "mutation", and the like, refer to any detectable change in genetic material, (*e.g.*, DNA), or any process, mechanism, or result of such a change. Mutations include gene mutations in which the structure (*e.g.*, DNA sequence) of the gene is altered; any DNA or other nucleic acid molecule derived from such a mutation process; and any expression product (*e.g.*,
30 the encoded protein) exhibiting a non-silent modification as a result of the mutation.

[0132] As used herein, the term "genetically modified animal" encompasses all animals into which an exogenous genetic material has been introduced and/or whose endogenous genetic material has been manipulated. Examples of genetically modified animals include without limitation transgenic animals, *e.g.*, "knock-in" animals with the endogenous gene substituted with a heterologous gene or an ortholog from another species or a mutated gene, "knockout" animals with the endogenous gene partially or completely inactivated, or transgenic animals expressing a mutated gene or overexpressing a wild-type or mutated gene (*e.g.*, upon targeted or random integration into the genome) and animals containing cells harboring a non-integrated nucleic acid construct (*e.g.*, viral-based vector, antisense oligonucleotide, shRNA, siRNA, ribozyme, etc.), including animals wherein the expression of an endogenous gene has been modulated (*e.g.*, increased or decreased) due to the presence of such construct.

[0133] As used herein, a "transgenic animal" is a nonhuman animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal include a transgene. Other examples of transgenic animals include nonhuman primates, sheep, dogs, pigs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA that is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal.

[0134] As used herein, a "homologous recombinant animal" is a nonhuman animal, preferably a mammal, more preferably a mouse, in which an endogenous gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

[0135] A "knock-in animal" is an animal (*e.g.*, a mammal such as a mouse or a rat) in which an endogenous gene has been substituted in part or in total with a heterologous gene (*i.e.*, a gene that is not endogenous to the locus in question; see Roamer *et al.*, *New Biol.* 1991, 3:331). This can be achieved by homologous

recombination (see “knockout animal” below), transposition (Westphal and Leder, *Curr. Biol.* 1997; 7: 530), use of mutated recombination sites (Araki *et al.*, *Nucleic Acids Res.* 1997; 25: 868), PCR (Zhang and Henderson, *Biotechniques* 1998; 25: 784), or any other technique known in the art. The heterologous gene may be, *e.g.*,
5 a reporter gene linked to the appropriate (*e.g.*, endogenous) promoter, which may be used to evaluate the expression or function of the endogenous gene (see, *e.g.*, Elegant *et al.*, *Proc. Natl. Acad. Sci. USA* 1998; 95: 11897).

[0136] A “knockout animal” is an animal (*e.g.*, a mammal such as a mouse or a rat) that has had a specific gene in its genome partially or completely
10 inactivated by gene targeting (see, *e.g.*, U.S. Patents Nos. 5,777,195 and 5,616,491). A knockout animal can be a heterozygous knockout (*i.e.*, with one defective allele and one wild type allele) or a homozygous knockout (*i.e.*, with both alleles rendered defective). Preparation of a knockout animal typically requires first introducing a nucleic acid construct (a “knockout construct”), that will be used to
15 decrease or eliminate expression of a particular gene, into an undifferentiated cell type termed an embryonic stem (ES) cell. The knockout construct is typically comprised of: (i) DNA from a portion (*e.g.*, an exon sequence, intron sequence, promoter sequence, or some combination thereof) of a gene to be knocked out; and (ii) a selectable marker sequence used to identify the presence of the knockout
20 construct in the ES cell. The knockout construct is typically introduced (*e.g.*, electroporated) into ES cells so that it can homologously recombine with the genomic DNA of the cell in a double crossover event. This recombined ES cell can be identified (*e.g.*, by Southern hybridization or PCR reactions that show the genomic alteration) and is then injected into a mammalian embryo at the blastocyst
25 stage. In a preferred embodiment where the knockout animal is a mammal, a mammalian embryo with integrated ES cells is then implanted into a foster mother for the duration of gestation (see, *e.g.*, Zhou *et al.*, *Genes and Dev.* 1995; 9: 2623-34).

[0137] The phrases “disruption of the gene”, “gene disruption”, and the
30 like, refer to: (i) insertion of a different or defective nucleic acid sequence into an endogenous (naturally occurring) DNA sequence, *e.g.*, into an exon or promoter

region of a gene; or (ii) deletion of a portion of an endogenous DNA sequence of a gene; or (iii) a combination of insertion and deletion, so as to decrease or prevent the expression of that gene or its gene product in the cell as compared to the expression of the endogenous gene sequence.

5 [0138] In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. See, *e.g.*, Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989 (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach, Volumes I and II* (Glover ed. 1985); *Oligonucleotide Synthesis* (Gait ed. 1984); *Nucleic Acid Hybridization* (Hames and Higgins eds. 1985); *Transcription And Translation* (Hames and Higgins eds. 1984); *Animal Cell Culture* (Freshney ed. 1986); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); Ausubel *et al.* eds., 15 *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. 1994; among others.

5.2. Polynucleotide Molecules

[0139] The present invention provides an isolated polynucleotide molecule comprising a nucleotide sequence encoding a mammalian PNPG5 protein. More particularly, the present invention provides an isolated polynucleotide molecule comprising a nucleotide sequence encoding a rat, mouse or human PNPG5 protein. In one embodiment, the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2. In another embodiment, the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO:4. In another embodiment, the human PNPG 25 protein comprises the amino acid sequence of SEQ ID NO:6.

[0140] In one embodiment, the amino acid sequence of the rat PNPG5 protein (SEQ ID NO: 2) is encoded by a polynucleotide molecule comprising the nucleotide sequence of SEQ ID NO:1 (cDNA sequence), or a degenerate variant thereof. In another embodiment, the amino acid sequence of the rat PNPG5 protein

is encoded by a polynucleotide molecule comprising the nucleotide sequence of the rat PNPG5 gene (SEQ ID NO:11), or a PNPG5-encoding portion thereof, or a degenerate variant thereof. The present invention also provides an isolated single-stranded polynucleotide molecule comprising a nucleotide sequence that is the complement of a nucleotide sequence of one strand of any of the aforementioned nucleotide sequences.

[0141] In one embodiment, the amino acid sequence of the mouse PNPG5 protein is encoded by a polynucleotide molecule comprising the nucleotide sequence of SEQ ID NO:3 (cDNA sequence), or a degenerate variant thereof. In another embodiment, the amino acid sequence of the mouse PNPG5 protein is encoded by a polynucleotide molecule comprising the nucleotide sequence of the PNPG5 gene (SEQ ID NO:12), or a PNPG5-encoding portion thereof, or a degenerate variant thereof. The present invention also provides an isolated single-stranded polynucleotide molecule comprising a nucleotide sequence that is the complement of a nucleotide sequence of one strand of any of the aforementioned nucleotide sequences.

[0142] In one embodiment, the amino acid sequence of the human PNPG5 protein is encoded by a polynucleotide molecule comprising the nucleotide sequence of SEQ ID NO:5 (cDNA sequence), or a degenerate variant thereof. In another embodiment, the amino acid sequence of the human PNPG5 protein is encoded by a polynucleotide molecule comprising the nucleotide sequence of the PNPG5 gene (SEQ ID NO:13) or a PNPG5-encoding portion thereof, or a degenerate variant thereof. The present invention also provides an isolated single-stranded polynucleotide molecule comprising a nucleotide sequence that is the complement of a nucleotide sequence of one strand of any of the aforementioned nucleotide sequences.

[0143] The present invention further provides an isolated polynucleotide molecule comprising a nucleotide sequence that hybridizes to the complement of a polynucleotide molecule comprising a nucleotide sequence that encodes the amino acid sequence of the rat, mouse or human PNPG5 protein of the present invention,

under moderately stringent conditions, such as, for example, an aqueous solution of 2×SSC at 65°C; alternatively, for example, hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C (see the Definitions section above). In a preferred embodiment, the homologous polynucleotide molecule hybridizes to the complement of a polynucleotide molecule comprising a nucleotide sequence that encodes the amino acid sequence of the rat, mouse or human PNPG5 protein of the present invention under highly stringent conditions, such as, for example, in an aqueous solution of 0.5×SSC at 65°C; alternatively, for example, hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% SDS 1 mM EDTA at 65°C, and washing in 0.1.x SSC/0.1% SDS at 68°C (see the Definitions Section 5.1., above). In a more preferred embodiment, the homologous polynucleotide molecule hybridizes under highly stringent conditions to the complement of a polynucleotide molecule consisting of a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.

[0144] The present invention further provides an isolated polynucleotide molecule comprising a nucleotide sequence that is homologous to the nucleotide sequence of a PNPG5-encoding polynucleotide molecule of the present invention. In a preferred embodiment, such a polynucleotide molecule hybridizes under standard conditions to the complement of a polynucleotide molecule comprising a nucleotide sequence that encodes the amino acid sequence of the rat, mouse or human PNPG5 protein of the present invention and has at least 75% sequence identity, preferably at least 80% sequence identity, more preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and most preferably at least 99% sequence identity to the nucleotide sequence of such PNPG5-encoding polynucleotide molecule (*e.g.*, as determined by a sequence comparison algorithm selected from BLAST, FASTA, DNA Strider, and GCG, and preferably as determined by the BLAST program from the National Center for Biotechnology Information (NCBI-Version 2.2), available on the WorldWideWeb at ncbi.nlm.nih.gov/BLAST/).

[0145] The present invention further provides an isolated polynucleotide molecule comprising a nucleotide sequence that encodes a polypeptide that is homologous to a rat, mouse or human PNPG5 protein of the present invention. Preferably, the polypeptides that are homologous to a PNPG5 protein of the present invention have the amino acid sequence identical to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, but have one or more amino acid residues conservatively substituted with a different amino acid residue. Conservative amino acid substitutions are well-known in the art. Rules for making such substitutions include those described by Dayhof, 1978, *Nat. Biomed. Res. Found.*, Washington, D.C., Vol. 5, Sup. 3, among others. More specifically, conservative amino acid substitutions are those that take place within a family of amino acids that are related in acidity, polarity, or bulkiness of their side chains. Genetically encoded amino acids are generally divided into four groups: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar=glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan and tyrosine are also jointly classified as aromatic amino acids. One or more replacements within any particular group, *e.g.*, of a leucine with an isoleucine or valine, or of an aspartate with a glutamate, or of a threonine with a serine, or of any other amino acid residue with a structurally related amino acid residue, *e.g.*, an amino acid residue with similar acidity, polarity, bulkiness of side chain, or with similarity in some combination thereof, will generally have an insignificant effect on the function or immunogenicity of the polypeptide.

[0146] In one embodiment of this invention, the amino acid sequences encoded by the homologous polynucleotide molecules above have the same function as the PNPG5 protein. These functions can include the promotion of neurite extension of hippocampal neurons, perturbing the development of fasciculated axon tracts, homophilic binding, and heterophilic binding to AMIGO2 and PNPG3/AMIGO3 proteins (Kuja-Panula *et al.*, *J. Cell Biol.* 2003, 160: 963-973). In a specific embodiment, the activity of the PNPG5 protein is its ability to interact

with a protein comprising an LRR domain such as, for example, a PNPG2 protein. In yet another specific embodiment, an activity of the PNPG5 protein is its ability to promote PNPG2-mediated signaling.

[0147] In one embodiment, the homologous polypeptide has at least about 5 75%, at least about 80%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 (*e.g.*, as determined by a sequence comparison algorithm selected from BLAST, FASTA, DNA Strider, and GCG, and preferably as determined by the BLAST program from the National Center for Biotechnology Information (NCBI-Version 2.2), available at 10 ncbi.nlm.nih.gov/BLAST/ on the WorldWideWeb). In another embodiment, the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 has been conservatively substituted at one, two, three, four or five non-conserved amino acid residue positions.

[0148] Also encompassed by the present invention are orthologs of the 15 specifically disclosed PNPG5 proteins and PNPG5-encoding nucleic acids. Additional PNPG5 orthologs can be identified based on the sequences of rat, mouse and human orthologs disclosed herein, using standard sequence comparison algorithms such as BLAST, FASTA, DNA Strider, GCG, etc. In addition to rat, mouse and human orthologs, particularly useful PNPG5 orthologs of the present 20 invention are monkey, dog, guinea pig, and porcine orthologs. As with the homologs discussed above, these orthologs can have the same functions as the PNPG5 protein.

[0149] The present invention further provides a polynucleotide molecule consisting of a nucleotide sequence that is a substantial portion of the nucleotide 25 sequence of any of the aforementioned PNPG5-related polynucleotide molecules of the present invention, or the complement of such nucleotide sequence. As used herein, a "substantial portion" of a PNPG5-encoding nucleotide sequence means a nucleotide sequence that is less than the nucleotide sequence required to encode a complete PNPG5 protein of the present invention, but comprising at least about 5%, 30 at least about 10%, at least about 20%, at least about 30%, at least about 40%, at

least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or at least about 99% of the contiguous nucleotide sequence of a PNPG5-encoding polynucleotide molecule of the present invention. Such polynucleotide molecules can be used for a variety of purposes including, *e.g.*,
5 to express a portion of a PNPG5 protein of the present invention in an appropriate expression system, or for use in conducting an assay to determine the expression level of a PNPG5 gene in a biological sample, or to amplify a PNPG5-encoding polynucleotide molecule.

[0150] In one embodiment, the present invention provides a soluble secreted
10 form of a PNPG5 protein, which protein is lacking a transmembrane domain, and a polynucleotide molecule having a nucleotide sequence encoding said soluble protein.

[0151] In addition to the nucleotide sequences of any of the aforementioned PNPG5-related polynucleotide molecules, polynucleotide molecules of the present invention can further comprise, or alternatively may consist of, nucleotide sequences
15 selected from those sequences that naturally flank a PNPG-encoding nucleotide sequence in the chromosome, including regulatory sequences.

[0152] The polynucleotide molecules encompassed by the present invention exclude uncharacterized clones in man-made genomic or cDNA libraries.

5.3. Oligonucleotides

20 [0153] The present invention further provides an oligonucleotide molecule that hybridizes to a polynucleotide molecule of the present invention, or that hybridizes to a polynucleotide molecule having a nucleotide sequence that is the complement of a nucleotide sequence of a polynucleotide molecule of the present invention. Such an oligonucleotide molecule: (i) is about 10 nucleotides to about
25 200 nucleotides in length, preferably from about 15 to about 100 nucleotides in length, and more preferably about 20 to about 50 nucleotides in length, and (ii) hybridizes to one or more of the polynucleotide molecules of the present invention under highly stringent conditions (*e.g.*, washing in 6x SSC/0.5% sodium pyrophosphate at about 37°C for about 14-base oligos, at about 48°C for about 17-

base oligos, at about 55°C for about 20-base oligos, and at about 60°C for about 23-base oligos). In one embodiment, an oligonucleotide molecule of the present invention is 100% complementary over its entire length to a portion of at least one of the aforementioned polynucleotide molecules of the present invention, and particularly any of SEQ ID NOS: 1, 3, 5, 11, 12, or 13. In another embodiment, an oligonucleotide molecule of the present invention is greater than 90% complementary over its entire length to a portion of at least one of the aforementioned polynucleotide molecules of the present invention, and particularly any of SEQ ID NOS: 1, 3, 5, 11, 12, or 13.

10 [0154] Specific non-limiting examples of oligonucleotide molecules according to the present invention include oligonucleotide molecules selected from the group consisting of SEQ ID NOS: 14-18 and 23-29 (listed in Table 2).

[0155] Oligonucleotide molecules of the present invention are useful for a variety of purposes, including as primers in amplification of a PNPG5-encoding polynucleotide molecule for use, *e.g.*, in differential diagnoses relating to pain conditions, or to encode or act as inhibitory molecules (*e.g.*, as antisense or short inhibitory (si) RNA molecules) useful in regulating expression of the PNPG5 gene product, or to identify PNPG5 orthologs in other species. Regarding diagnostics, suitably designed oligonucleotide primers (*e.g.*, PCR primers) and hybridization probes can be used to detect the presence and quantity of a PNPG5-specific polynucleotide molecule in a biological sample (*e.g.*, tissue or fluid, such as central nervous system (CNS) or peripheral nervous system (PNS) tissue, lung tissue, placental tissue, blood, cerebrospinal fluid, mucous, urine, amniotic fluid, etc) collected from a subject. According to the methods of the present invention, detection of particular levels of a PNPG5-encoding nucleic acid in a sample can be used for a diagnosis of a pain state in a subject. In addition, such a diagnostic approach can be used to monitor the efficacy of an analgesic treatment or to determine the ability of a compound to modulate expression of the PNPG5-encoding nucleic acid.

[0156] Oligonucleotide molecules can be labeled, *e.g.*, with radioactive labels (*e.g.*, $\gamma^{32}\text{P}$), biotin, fluorescent labels, etc. In one embodiment, a labeled oligonucleotide molecule can be used as a probe to detect the presence of a nucleic acid. In another embodiment, two oligonucleotide molecules (one or both of which
5 may be labeled) can be used as PCR primers, either for cloning a full-length nucleic acid or a fragment of a nucleic acid encoding a gene product of interest, or to detect the presence of nucleic acids encoding a gene product. Methods for conducting amplifications, such as the polymerase chain reaction (PCR), are described, among other places, in Saiki *et al.*, *Science* 1988, 239:487 and U.S. Patent No. 4,683,202.
10 Other amplification techniques known in the art, *e.g.*, the ligase chain reaction, can alternatively be used (see, *e.g.*, U.S. Patent Nos. 6,335,184 and 6,027,923; Reyes *et al.*, *Clinical Chemistry* 2001; 47: 131-40; and Wu *et al.*, *Genomics* 1989; 4: 560-569).

[0157] In a further embodiment, an oligonucleotide molecule of the present
15 invention can form a triple helix with a PNPG5-encoding nucleic acid molecule, thereby inhibiting PNPG5 expression. In still another embodiment, an oligonucleotide molecule can be a short interfering or small hairpin RNA, or an antisense oligonucleotide, useful to inhibit expression of the PNPG5 gene. Generally, oligonucleotide molecules are prepared synthetically, preferably on a
20 nucleic acid synthesizer, and may be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, where appropriate.

5.4. Recombinant Expression Systems

5.4.1. Cloning and Expression Vectors

[0158] The present invention further provides compositions and constructs
25 for cloning and expressing any of the polynucleotide molecules of the present invention, including cloning vectors, expression vectors, transformed host cells comprising any of said vectors, and novel strains or cell lines derived therefrom. In one embodiment, the present invention provides a recombinant vector comprising a polynucleotide molecule having a nucleotide sequence encoding a mammalian
30 PNPG5 protein. In one embodiment, the mammalian PNPG5 protein is a rat,

mouse or human PNPG5 protein. In a specific embodiment, the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2. In another specific embodiment, the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO:4. In another specific embodiment, the human PNPG5 protein comprises an amino acid sequence of SEQ ID NO:6. In one embodiment, the polynucleotide molecule has the nucleotide sequence of SEQ ID NO: 1, 3, or 5, or a degenerate variant thereof.

[0159] In one specific non-limiting embodiment, the present invention provides plasmid pPNPG5 (ATCC Accession No. PTA-5808); deposited with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, VA 20110-2209, USA on February 9, 2004, which comprises a polynucleotide molecule having a nucleotide sequence encoding the rat PNPG5 protein.

[0160] Recombinant vectors of the present invention, particularly expression vectors, are preferably constructed so that the coding sequence for the polynucleotide molecule of the present invention is in operative association with one or more regulatory elements necessary for transcription and translation of the coding sequence to produce a polypeptide. As used herein, the term "regulatory element" includes but is not limited to nucleotide sequences that encode inducible and non-inducible promoters, enhancers, operators and other elements known in the art that serve to drive and/or regulate expression of polynucleotide coding sequences. Also, as used herein, the coding sequence is in operative association with one or more regulatory elements where the regulatory elements effectively regulate and allow for the transcription of the coding sequence or the translation of its mRNA, or both.

[0161] Methods are known in the art for constructing recombinant vectors containing particular coding sequences in operative association with appropriate regulatory elements, and these can be used to practice the present invention. These methods include *in vitro* recombinant techniques, synthetic techniques, and *in vivo* genetic recombination. See, *e.g.*, the techniques described in Ausubel *et al.*, 1989, above; Sambrook *et al.*, 1989, above; Saiki *et al.*, 1988, above; Reyes *et al.*, 2001,

above; Wu *et al.*, 1989, above; U.S. Patent Nos. 4,683,202; 6,335,184 and 6,027,923.

[0162] A variety of expression vectors are known in the art that can be utilized to express a polynucleotide molecule of the present invention, including
5 recombinant bacteriophage DNA, plasmid DNA, and cosmid DNA expression vectors containing the particular coding sequences. Typical prokaryotic expression vector plasmids that can be engineered to contain a polynucleotide molecule of the present invention include pUC8, pUC9, pBR322 and pBR329 (Biorad Laboratories, Richmond, CA), pPL and pKK223 (Pharmacia, Piscataway, NJ), pQE50 (Qiagen,
10 Chatsworth, CA), and pGEM-T EASY (Promega, Madison, WI), pcDNA6.2/V5-DEST and pcDNA3.2/V5DEST (Invitrogen, Carlsbad, CA) among many others. Typical eukaryotic expression vectors that can be engineered to contain a polynucleotide molecule of the present invention include an ecdysone-inducible mammalian expression system (Invitrogen, Carlsbad, CA), cytomegalovirus
15 promoter-enhancer-based systems (Promega, Madison, WI; Stratagene, La Jolla, CA; Invitrogen), and baculovirus-based expression systems (Promega), among many others.

[0163] The regulatory elements of these and other vectors can vary in their strength and specificities. Depending on the host/vector system utilized, any of a
20 number of suitable transcription and translation elements can be used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, *e.g.*, mouse metallothionein promoter, or from viruses that grow in these cells, *e.g.*, vaccinia virus 7.5 K promoter or Maloney murine sarcoma virus long terminal repeat, can be used. Promoters obtained by recombinant DNA or
25 synthetic techniques can also be used to provide for transcription of the inserted sequence. In addition, expression from certain promoters can be elevated in the presence of particular inducers, *e.g.*, zinc and cadmium ions for metallothionein promoters. Non-limiting examples of transcriptional regulatory regions or promoters include for bacteria, the β -gal promoter, the T7 promoter, the TAC
30 promoter, λ left and right promoters, trp and lac promoters, trp-lac fusion promoters, etc.; for yeast, glycolytic enzyme promoters, such as ADH-I and -II

promoters, GPK promoter, PGI promoter, TRP promoter, etc.; and for mammalian cells, SV40 early and late promoters, and adenovirus major late promoters, among others.

[0164] Specific initiation signals are also required for sufficient translation of inserted coding sequences. These signals typically include an ATG initiation codon and adjacent sequences. In cases where the polynucleotide molecule of the present invention, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translation control signals may be needed. However, in cases where only a portion of a coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, may be required. These exogenous translational control signals and initiation codons can be obtained from a variety of sources, both natural and synthetic. Furthermore, the initiation codon must be in-phase with the reading frame of the coding regions to ensure in-frame translation of the entire insert.

[0165] Expression vectors can also be constructed that will express a fusion protein comprising a protein or polypeptide of the present invention. Such fusion proteins can be used, *e.g.*, to raise anti-sera against a PNPG5 protein, to study the biochemical properties of the PNPG5 protein, to engineer a variant of a PNPG5 protein exhibiting different immunological or functional properties, or to aid in the identification or purification, or to improve the stability, of a recombinantly-expressed PNPG5 protein. Possible fusion protein expression vectors include but are not limited to vectors incorporating sequences that encode β -galactosidase and *trpE* fusions, maltose-binding protein fusions, glutathione-S-transferase fusions, polyhistidine fusions (carrier regions), V5, HA, myc, and HIS. Methods known in the art can be used to construct expression vectors encoding these and other fusion proteins.

[0166] The fusion protein can be useful to aid in purification of the expressed protein. In non-limiting embodiments, *e.g.*, a PNPG5-maltose-binding fusion protein can be purified using amylose resin; a PNPG5-glutathione-S-transferase fusion protein can be purified using glutathione-agarose beads; and a

PNPG5-polyhistidine fusion protein can be purified using divalent nickel resin. Alternatively, antibodies against a carrier protein or peptide can be used for affinity chromatography purification of the fusion protein. For example, a nucleotide sequence coding for the target epitope of a monoclonal antibody can be engineered
5 into the expression vector in operative association with the regulatory elements and situated so that the expressed epitope is fused to a PNP5 protein of the present invention. In a non-limiting embodiment, a nucleotide sequence coding for the FLAGTM epitope tag (International Biotechnologies Inc.), which is a hydrophilic marker peptide, can be inserted by standard techniques into the expression vector at
10 a point corresponding, *e.g.*, to the amino or carboxyl terminus of the PNP5 protein. The expressed PNP5 protein-FLAGTM epitope fusion product can then be detected and affinity-purified using commercially available anti-FLAGTM antibodies. The expression vector can also be engineered to contain polylinker sequences that encode specific protease cleavage sites so that the expressed PNP5 protein can be
15 released from a carrier region or fusion partner by treatment with a specific protease. For example, the fusion protein vector can include a nucleotide sequence encoding a thrombin or factor Xa cleavage site, among others.

[0167] A signal sequence upstream from, and in reading frame with, the PNP5 coding sequence can be engineered into the expression vector by known
20 methods to direct the trafficking and secretion of the expressed protein. Non-limiting examples of signal sequences include those from α -factor, immunoglobulins, outer membrane proteins, penicillinase, and T-cell receptors, among others.

[0168] To aid in the selection of host cells transformed or transfected with a
25 recombinant vector of the present invention, the vector can be engineered to further comprise a coding sequence for a reporter gene product or other selectable marker. Such a coding sequence is preferably in operative association with the regulatory elements, as described above. Reporter genes that are useful in practicing the invention are known in the art, and include those encoding chloramphenicol
30 acetyltransferase (CAT), green fluorescent protein, firefly luciferase, and human growth hormone, among others. Nucleotide sequences encoding selectable markers

are known in the art, and include those that encode gene products conferring resistance to antibiotics or anti-metabolites, or that supply an auxotrophic requirement. Examples of such sequences include those that encode thymidine kinase activity, or resistance to methotrexate, ampicillin, kanamycin, chloramphenicol, zeocin, pyrimethamine, aminoglycosides, hygromycin, blasticidine, or neomycin, among others.

5.4.2. Transformation of Host Cells

[0169] The present invention further provides a transformed host cell comprising a polynucleotide molecule or recombinant vector of the present invention, and a cell line derived therefrom. Such host cells are useful for cloning and/or expressing a polynucleotide molecule of the present invention. Such transformed host cells include but are not limited to microorganisms, such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA vectors, or yeast transformed with a recombinant vector, or animal cells, such as insect cells infected with a recombinant virus vector, *e.g.*, baculovirus, or mammalian cells infected with a recombinant virus vector, *e.g.*, adenovirus, vaccinia virus, lentivirus, adeno-associated virus (AAV), or herpesvirus, among others. For example, a strain of *E. coli* can be used such as, *e.g.*, the DH5 α strain available from the ATCC, Manassas, VA, USA (Accession No. 31343), or from Stratagene (La Jolla, CA). Eukaryotic host cells include yeast cells, although mammalian cells, *e.g.*, from a mouse, rat, hamster, cow, monkey, or human cell line, among others, can also be utilized effectively. Examples of eukaryotic host cells that can be used to express a recombinant protein of the invention include Chinese hamster ovary (CHO) cells (*e.g.*, ATCC Accession No. CCL-61), NIH Swiss mouse embryo cells NIH/3T3 (*e.g.*, ATCC Accession No. CRL-1658), human epithelial kidney cells HEK 293 (*e.g.*, ATCC Accession No. CRL-1573), and Madin-Darby bovine kidney (MDBK) cells (ATCC Accession No. CCL-22).

[0170] As described above, the present invention provides for mammalian cells infected with a virus containing a recombinant viral vector of the present

invention. For example, an overview and instructions concerning the infection of mammalian cells with adenovirus using the AdEasy™ Adenoviral Vector System is given in the Instructions Manual for this system from Stratagene (La Jolla, CA). As another example, an overview and instructions concerning the infection of mammalian cells with AAV using the AAV Helper-Free System is given in the
5 Instructions Manual for this system from Stratagene (La Jolla, CA).

[0171] The recombinant vector of the invention is preferably transformed or transfected into one or more host cells of a substantially homogeneous culture of cells. The vector is generally introduced into host cells in accordance with known
10 techniques, such as, *e.g.*, by protoplast transformation, calcium phosphate precipitation, calcium chloride treatment, microinjection, electroporation, transfection by contact with a recombined virus, liposome-mediated transfection, DEAE-dextran transfection, transduction, conjugation, or microprojectile bombardment, among others. Selection of transformants can be conducted by
15 standard procedures, such as by selecting for cells expressing a selectable marker, *e.g.*, antibiotic resistance, associated with the recombinant expression vector.

[0172] Once an expression vector is introduced into the host cell, the presence of the polynucleotide molecule of the present invention, either integrated into the host cell genome or maintained episomally, can be confirmed by standard
20 techniques, *e.g.*, by DNA-DNA, DNA-RNA, or RNA-antisense RNA hybridization analysis, restriction enzyme analysis, PCR analysis including reverse transcriptase PCR (RT-PCR), detecting the presence of a "marker" gene function, or by immunological or functional assay to detect the expected protein product.

5.4.3. Expression and Purification of Recombinant Polypeptides

25 [0173] Once a polynucleotide molecule of the present invention has been stably introduced into an appropriate host cell, the transformed host cell is clonally propagated, and the resulting cells can be grown under conditions conducive to the efficient production (*i.e.*, expression or overexpression) of the encoded polypeptide. Where the expression vector comprises an inducible promoter, appropriate induction

conditions such as, *e.g.*, temperature shift, exhaustion of nutrients, addition of gratuitous inducers (*e.g.*, analogs of carbohydrates, such as isopropyl- β -D-thiogalactopyranoside (IPTG)), accumulation of excess metabolic by-products, or the like, are employed as needed to induce expression. If necessary and desired, a
5 signal sequence that matches with a host cell can be added to the N-terminus of the polypeptide. Examples of the signal sequences that can be used are PhoA signal sequence, OmpA signal sequence, etc., in the case of using bacteria of the genus *Escherichia* as the host; α -amylase signal sequence, subtilisin signal sequence, etc., in the case of using bacteria of the genus *Bacillus* as the host; MF α signal sequence,
10 SUC2 signal sequence, etc., in the case of using yeast as the host; and insulin signal sequence, α -interferon signal sequence, antibody molecule signal sequence, etc., in the case of using animal cells as the host, respectively.

[0174] Where the polypeptide is retained inside the host cells or contained in a cell membrane, the cells are harvested and lysed, and the product is substantially
15 purified or isolated from the lysate or membrane fraction under extraction conditions known in the art to minimize protein degradation such as, *e.g.*, at 4°C, or in the presence of protease inhibitors, or both. Where the polypeptide is secreted from the host cells, the exhausted nutrient medium can simply be collected and the polypeptide substantially purified or isolated therefrom.

20 [0175] The polypeptide can be substantially purified or isolated from cell lysates, membrane fractions, or culture medium, as necessary, using standard methods, including but not limited to one or more of the following methods: ammonium sulfate precipitation, size fractionation, ion exchange chromatography, HPLC, density centrifugation, affinity chromatography, ethanol precipitation, and
25 chromatofocusing. During purification, the polypeptide can be detected based, *e.g.*, on size, or reactivity with a polypeptide-specific antibody, or by detecting the presence of a fusion tag.

[0176] According to the present invention, the recombinantly expressed full-length PNPG5 protein is most likely to be associated with the cellular membrane as
30 a transmembrane protein. Such protein can be isolated from membrane fractions of

host cells. The cell membrane fraction refers to a fraction abundant in cell membrane obtained by cell disruption and subsequent fractionation by any of the known methods. Useful cell disruption methods include, *e.g.*, cell squashing using a Potter-Elvehjem homogenizer, disruption using a Waring blender or Polytron (manufactured by Kinematica Inc.), disruption by ultrasonication, and disruption by cell spraying through thin nozzles under an increased pressure using a French press or the like. Cell membrane fractionation is effected mainly by fractionation using a centrifugal force, such as centrifugation for fractionation and density gradient centrifugation. For example, cell disruption fluid can be centrifuged at a low speed (500 rpm to 3,000 rpm) for a short period of time (normally about 1 to about 10 minutes), the resulting supernatant is then centrifuged at a higher speed (15,000 rpm to 30,000 rpm) normally for 30 minutes to 2 hours. The precipitate thus obtained can be used as the membrane fraction. The membrane fraction is rich in membrane components such as cell-derived phospholipids and transmembrane and membrane-associated proteins. In yet other embodiments, the membrane fraction may be further solubilized with a detergent. Detergents that may be used with the present invention include without limitation Triton X-100, β -octyl glucoside, and CHAPS (see also Langridge *et al.*, *Biochim. Biophys. Acts.* 1983; 751: 318).

[0177] A preferred method for isolating transmembrane proteins is a technique that uses 2-D gel electrophoresis as described, for example, in the Instructions for "2-D Sample Prep for Membrane Proteins" from Pierce Biotechnology, Inc. (Rockford, IL).

[0178] Upon isolation of the membrane fraction, the peripheral proteins of these membranes can be removed by extraction with high salt concentrations, high pH or chaotropic agents such as lithium diiodosalicylate. The integral proteins (including the PNPG5 protein) can then be solubilized using a detergent such as Triton X-100, β -octyl glucoside, CHAPS, or other compounds of similar action (see, *e.g.*, Beros *et al.*, *J. Biol. Chem.* 1987; 262: 10613). A combination of several standard chromatographic steps (*e.g.*, ion exchange chromatography, gel permeation chromatography, adsorption chromatography or isoelectric focusing) and/or a single purification step involving immuno-affinity chromatography using

immobilized antibodies (or antibody fragments) to the PNPG5 protein and/or preparative polyacrylamide gel electrophoresis using instrumentation such as the Applied Biosystems "230A EPEC System" can be then used to purify the PNPG5 protein and remove it from other integral proteins of the detergent-stabilized mixture. It is recognized that the hydrophobic nature of the transmembrane protein may necessitate the inclusion of amphiphilic compounds such as detergents and other surfactants (see bud Kar and Maloney, *J. Biol. Chem.* 1986; 261: 10079) during handling.

[0179] For use in practicing the present invention, the polypeptide can be in an unpurified state as secreted into the culture fluid or as present in a cell lysate or membrane fraction. Alternatively, the polypeptide may be purified therefrom. Once a polypeptide of the present invention of sufficient purity has been obtained, it can be characterized by standard methods, including by SDS-PAGE, size exclusion chromatography, amino acid sequence analysis, immunological activity, biological activity, etc. The polypeptide can be further characterized using hydrophilicity analysis (see, *e.g.*, Hopp and Woods, *Proc. Natl. Acad. Sci. USA* 1981; 78: 3824), or analogous software algorithms, to identify hydrophobic and hydrophilic regions. Structural analysis can be carried out to identify regions of the polypeptide that assume specific secondary structures. Biophysical methods such as X-ray crystallography (Engstrom, *Biochem. Exp. Biol.* 1974; 11: 7-13), computer modeling (Fletterick and Zoller eds., *In: Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), and nuclear magnetic resonance (NMR) can be used to map and study potential sites of interaction between the polypeptide and other putative interacting proteins/receptors/molecules. Information obtained from these studies can be used to design deletion mutants, and to design or select therapeutic compounds that can specifically modulate the biological function of the PNPG5 protein *in vivo*.

5.5. Polypeptides

[0180] The present invention further provides an isolated polypeptide encoded by a polynucleotide of the present invention, which polypeptide may or may not also be purified.

5 [0181] In one embodiment, the isolated polypeptide is a rat PNPG5 protein comprising the amino acid sequence of SEQ ID NO:2. In another embodiment, the isolated polypeptide is a mouse PNPG5 protein comprising the amino acid sequence of SEQ ID NO:4. In another embodiment, the isolated polypeptide is a human PNPG5 protein comprising the amino acid sequence of SEQ ID NO:6.

10 [0182] The present invention further provides a polypeptide that is homologous to a rat, mouse or human PNPG5 protein of the present invention, as the term "homologous" is defined above for polynucleotides.

[0183] The present invention further provides a polypeptide consisting of a substantial portion of a rat, mouse or human PNPG5 protein of the present invention. As used herein, a "substantial portion" (also referred to as a "peptide fragment") of such a protein refers to a polypeptide consisting of less than the complete amino acid sequence of the corresponding full-length protein, but comprising at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or at least about 99% of the contiguous amino acid sequence of the full-length protein. Such peptide fragments are useful if they either exhibit or inhibit PNPG5 activity or are immunogenic, *i.e.*, capable of inducing an immune response resulting in the production of antibodies that react specifically against the corresponding full-length PNPG5 polypeptide.

25 [0184] In a specific embodiment, the invention provides an isolated soluble secreted PNPG5 polypeptide lacking the transmembrane domain of the full length protein.

[0185] In one embodiment of this invention, the homologous polypeptides or substantial portions of PNPG5 protein above have the same function as the PNPG5 protein. These functions can include the promotion of neurite extension of hippocampal neurons, perturbing the development of fasciculated axon tracts, homophilic binding, and heterophilic binding to AMIGO2 and PNPG3/AMIGO3 proteins (Kuja-Panula *et al.*, *J. Cell Biol.* 2003, 160: 963-973). In a specific embodiment, the activity of the PNPG5 protein is its ability to interact with a protein comprising an LRR domain such as, for example, a PNPG2 protein. In yet another specific embodiment, an activity of the PNPG5 protein is its ability to promote PNPG2-mediated signaling.

[0186] The present invention further provides fusion proteins comprising any of the aforementioned polypeptides (proteins or peptide fragments) fused to a carrier or fusion partner, as known in the art. For example, PNPG5 can be fused with green fluorescent protein (GFP), V5, and Ig.

[0187] Polypeptides of the present invention are useful for a variety of purposes, including for use in cell-based or non-cell-based assays to study the biological function of PNPG5, to identify molecules that interact with PNPG5 relevant to its *in vivo* function, to screen for compounds that bind to PNPG5 and modulate its expression and/or activity, or as antigens to raise polyclonal or monoclonal antibodies, as described below. Such antibodies can be used as therapeutic agents to modulate the activity of PNPG5 activity, or as diagnostic reagents, *e.g.*, using standard techniques such as Western blot assays or immunostaining, to screen for PNPG5 protein expression levels in cell, tissue or fluid samples collected from a subject.

[0188] In a specific embodiment, polypeptides of the present invention can be used to determine their inhibitory or stimulatory effect on cellular functions or growth. As described in Kuja-Panula *et al.* (*J. Cell Biol.* 2003, 160: 963-973), immobilized AMIGO-Ig fusion proteins promote neurite extension of hippocampal neurons. Additionally, soluble AMIGO-Ig fusion proteins perturb development of fasciculated axon tracts *in vitro*.

[0189] A polypeptide of the present invention can be modified at the protein level to improve or otherwise alter its biological or immunological characteristics. One or more chemical modifications of the polypeptide can be carried out using known techniques to prepare analogs therefrom, including but not limited to any of the following: substitution of one or more L-amino acids of the polypeptide with corresponding D-amino acids, amino acid analogs, or amino acid mimics, so as to produce, *e.g.*, carbazates or tertiary centers; or specific chemical modification, such as, *e.g.*, proteolytic cleavage with trypsin, chymotrypsin, papain or V8 protease, or treatment with NaBH₄ or cyanogen bromide, or acetylation, formylation, oxidation or reduction, etc. Alternatively or additionally, a polypeptide of the present invention can be modified by genetic recombination techniques.

[0190] A polypeptide of the present invention can be derivatized by conjugation thereto of one or more chemical groups, including but not limited to acetyl groups, sulfur bridging groups, glycosyl groups, lipids, and phosphates, and/or by conjugation to a second polypeptide of the present invention, or to another protein, such as, *e.g.*, serum albumin, keyhole limpet hemocyanin, or commercially available BSA, or to a polyamino acid (*e.g.*, polylysine), or to a polysaccharide, (*e.g.*, sepharose, agarose, or modified or unmodified celluloses), among others. Such conjugation is preferably by covalent linkage at amino acid side chains and/or at the N-terminus or C-terminus of the polypeptide. Methods for carrying out such conjugation reactions are known in the field of protein chemistry.

[0191] Derivatives useful in practicing the claimed invention also include those in which a water-soluble polymer such as, *e.g.*, polyethylene glycol, is conjugated to a polypeptide of the present invention, or to an analog or derivative thereof, thereby providing additional desirable properties while retaining, at least in part, the immunogenicity of the polypeptide. These additional desirable properties include, *e.g.*, increased solubility in aqueous solutions, increased stability in storage, increased resistance to proteolytic degradation, and increased *in vivo* half-life. Water-soluble polymers suitable for conjugation to a polypeptide of the present invention include but are not limited to polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene

glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, polyvinyl ethyl ethers, and α,β -poly[2-hydroxyethyl]-DL-aspartamide. Polyethylene glycol is particularly preferred. Methods for making water-soluble polymer conjugates of polypeptides are known in the art and are described, among other places, in U.S. Patent Nos. 3,788,948; 3,960,830; 4,002,531; 4,055,635; 4,179,337; 4,261,973; 4,412,989; 4,414,147; 4,415,665; 4,609,546; 4,732,863; and 4,745,180; European Patent (EP) 152,847; EP 98,110; and Japanese Patent 5,792,435; which patents are incorporated herein by reference.

5.6. Antibodies

[0192] As used herein, the term “antibody” refers to a human, nonhuman, or chimeric (*e.g.*, humanized) immunoglobulin, or binding fragment thereof, that specifically binds to an antigen, *e.g.*, a PNPG5 protein. Suitable antibodies may be polyclonal (*e.g.*, sera or affinity purified preparations), monoclonal, or recombinant. Examples of useful fragments include separate heavy chains, light chains, Fab, F(ab')₂, Fabc, and Fv fragments. Fragments can be produced by enzymatic or chemical separation of intact immunoglobulins or by recombinant DNA techniques. Fragments may be expressed in the form of phage-coat fusion proteins (see, *e.g.*, International PCT Publication Nos. WO 91/17271, WO 92/01047, and WO 92/06204). Typically, the antibodies, fragments, or similar binding agents bind a specific antigen with an affinity of at least 10^7 , 10^8 , 10^9 , or 10^{10} M^{-1} .

[0193] The present invention provides an isolated antibody directed against a polypeptide of the present invention. In a specific embodiment, antibodies can be raised against a PNPG5 protein of the invention using known methods in view of this disclosure. Various host animals selected, *e.g.*, from pigs, cows, horses, rabbits, goats, sheep, rats, or mice, can be immunized with a partially or substantially purified PNPG5 protein, or with a peptide homolog, fusion protein, peptide fragment, analog or derivative thereof, as described above. An adjuvant can be used to enhance antibody production.

[0194] Polyclonal antibodies can be obtained and isolated from the serum of an immunized animal and tested for specificity against the antigen using standard techniques. Alternatively, monoclonal antibodies can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, *Nature* 1975; 256: 495-497; the human B-cell hybridoma technique (Kosbor *et al.*, *Immunology Today* 1983; 4: 72; Cote *et al.*, *Proc. Natl. Acad. Sci. USA* 1983; 80: 2026-2030); and the EBV-hybridoma technique (Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., 1985, pp. 77-96). Alternatively, techniques described for the production of single chain antibodies (see, *e.g.*, U.S. Patent No. 4,946,778) can be adapted to produce PNPG5-specific single chain antibodies.

[0195] Antibody fragments that contain specific binding sites for a polypeptide of the present invention are also encompassed within the present invention, and can be generated by known techniques. Such fragments include but are not limited to F(ab')₂ fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries can be constructed (Huse *et al.*, *Science* 1989; 246: 1275-1281) to allow rapid identification of Fab fragments having the desired specificity to the particular PNPG5 protein.

[0196] Techniques for the production and isolation of monoclonal antibodies and antibody fragments are known in the art, and are generally described, among other places, in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, and in Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, London, 1986.

[0197] Antibodies or antibody fragments can be used in methods known in the art relating to the localization and activity of PNPG5, *e.g.*, in Western blotting, *in situ* imaging, measuring levels thereof in appropriate physiological samples, etc. Immunoassay techniques using antibodies include radioimmunoassay, ELISA

(enzyme-linked immunosorbant assay), “sandwich” immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, *in situ* immunoassays (using, *e.g.*, colloidal gold, enzyme or radioisotope labels), precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. Antibodies can also be used in microarrays (see, *e.g.*, International PCT Publication No. WO 00/04389). Furthermore, antibodies can be used as therapeutics to inhibit the activity of a PNPG5 protein.

[0198] Recent advances in antibody engineering have allowed the genes encoding antibodies to be manipulated, so that antigen-binding molecules can be expressed within mammalian cells. Application of gene technologies to antibody engineering has enabled the synthesis of single-chain fragment variable (scFv) antibodies that combine within a single polypeptide chain the light and heavy chain variable domains of an antibody molecule covalently joined by a pre-designed peptide linker. Intracellular antibody (or “intrabody”) strategy serves to target molecules involved in essential cellular pathways for modification or ablation of protein function. Antibody genes for intracellular expression can be derived, *e.g.*, either from murine or human monoclonal antibodies or from phage display libraries. For intracellular expression, small recombinant antibody fragments containing the antigen recognizing and binding regions can be used. Intrabodies can be directed to different intracellular compartments by targeting sequences attached to the antibody fragments.

[0199] Various methods have been developed to produce intrabodies. Techniques described for the production of single chain antibodies (see, *e.g.*, U.S. Patents No. 5,476,786; 5,132,405; and 4,946,778) can be adapted to produce polypeptide-specific single chain antibodies. Another method called intracellular antibody capture (IAC), is based on a genetic screening approach (Tanaka *et al.*, *Nucleic Acids Res.* 2003; 31: e23). Using this technique, consensus immunoglobulin variable frameworks are identified that can form the basis of intrabody libraries for direct screening. The procedure comprises *in vitro*

production of a single antibody gene fragment from oligonucleotides and diversification of CDRs of the immunoglobulin variable domain by mutagenic PCR to generate intrabody libraries. This method obviates the need for *in vitro* production of antigen for pre-selection of antibody fragments, and also yields
5 intrabodies with enhanced intracellular stability.

[0200] Intrabodies can be used to modulate cellular physiology and metabolism through a variety of mechanisms, including blocking, stabilizing, or mimicking protein-protein interactions, by altering enzyme function, or by diverting proteins from their usual intracellular compartments. Intrabodies can be directed to
10 the relevant cellular compartments by modifying the genes that encode them to specify N- or C-terminal polypeptide extensions for providing intracellular-trafficking signals.

5.7. Targeted Mutation of the PNPG5 Gene

[0201] Based on the present disclosure of polynucleotide molecules, genetic
15 constructs can be prepared for use in disabling or otherwise mutating a mammalian PNPG5 gene. For example, the rat, mouse or human PNPG5 gene can be mutated using an appropriately designed genetic construct in combination with genetic techniques currently known or to be developed in the future. For example, a rat, mouse or human PNPG5 gene can be mutated using a genetic construct that
20 functions to: (a) delete all or a portion of the coding sequence or regulatory sequence of the PNPG5 gene; or (b) replace all or a portion of the coding sequence or regulatory sequence of the PNPG5 gene with a different nucleotide sequence; or (c) insert into the coding sequence or regulatory sequence of the PNPG5 gene one or more nucleotides, or an oligonucleotide molecule, or polynucleotide molecule,
25 which can comprise a nucleotide sequence from the same species or from a heterologous source; or (d) carry out some combination of (a), (b) and (c).

[0202] Cells, tissues and animals in which the PNPG5 gene has been mutated are useful, among others, in studying the biological function of PNPG5, identifying molecules that interact with PNPG5 relevant to its *in vivo* function, as

well as in conducting screens to identify therapeutic compounds that selectively modulate PNPG5 expression and/or activity. In a preferred embodiment, the mutation serves to partially or completely disable the PNPG5 gene, or partially or completely disable the protein encoded by the PNPG5 gene. In this context, a
5 PNPG5 gene or protein is considered to be partially or completely disabled if either no protein product is made (for example, where the gene is deleted), or a protein product is made that can no longer carry out its normal biological function or can no longer be transported to its normal cellular location, or a protein product is made that carries out its normal biological function but at a significantly reduced level.

10 [0203] In a non-limiting embodiment, a genetic construct of the present invention is used to mutate a wild-type PNPG5 gene by replacement of at least a portion of the coding or regulatory sequence of the wild-type gene with a different nucleotide sequence such as, *e.g.*, a mutated coding sequence or mutated regulatory region, or portion thereof. A mutated PNPG5 gene sequence for use in such a
15 genetic construct can be produced by any of a variety of known methods, including by use of error-prone PCR, or by cassette mutagenesis. For example, oligonucleotide-directed mutagenesis can be employed to alter the coding or regulatory sequence of a wild-type PNPG5 gene in a defined way, *e.g.*, to introduce a frame-shift or a termination codon at a specific point within the sequence. A
20 mutated nucleotide sequence for use in the genetic construct of the present invention can be prepared by insertion into the coding or regulatory (*e.g.*, promoter) sequence of one or more nucleotides, oligonucleotide molecules or polynucleotide molecules, or by replacement of a portion of the coding sequence or regulatory sequence with one or more different nucleotides, oligonucleotide molecules or polynucleotide
25 molecules. Such oligonucleotide molecules or polynucleotide molecules can be obtained from any naturally occurring source or can be synthetic. The inserted sequence can serve simply to disrupt the reading frame of the PNPG5 gene, or can further encode a heterologous gene product such as a selectable marker.

[0204] In one embodiment, the leucine-rich repeat (LRR) domains and/or the
30 IG domain of PNPG5 (see **Figure 4**) can be mutated. These mutations and other mutations can be useful in determining the biological activity of PNPG5, the

interactions between PNPG5 and amphoterin, the binding interactions between PNPG5 and either AMIGO2 or PNPG3/AMIGO3, and the interactions between PNPG5 and PNPG2.

[0205] Mutations to produce modified cells, tissues and animals that are
5 useful in practicing the present invention can occur anywhere in the PNPG5 gene,
including in the open reading frame, or in the promoter or other regulatory region,
or in any other portion of the sequence that naturally comprises the gene or ORF.
Such cells include mutants in which a modified form of the PNPG5 protein normally
encoded by the PNPG5 gene is produced, or in which no protein normally encoded
10 by the PNPG5 gene is produced. Such cells can be null, conditional or leaky
mutants.

[0206] Alternatively, a genetic construct can comprise nucleotide sequences
that naturally flank the PNPG5 gene or ORF *in situ*, with only a portion or no
nucleotide sequences from the actual coding region of the gene itself. Such a
15 genetic construct can be useful to delete the entire PNPG5 gene or ORF.

[0207] Methods for carrying out homologous gene replacement are known in
the art. For targeted gene mutation through homologous recombination, the genetic
construct is preferably a plasmid, either circular or linearized, comprising a mutated
nucleotide sequence as described above. In a non-limiting embodiment, at least
20 about 200 nucleotides of the mutated sequence are used to specifically direct the
genetic construct of the present invention to the particular targeted PNPG5 gene for
homologous recombination, although shorter lengths of nucleotides may also be
effective. In addition, the plasmid preferably comprises an additional nucleotide
sequence encoding a reporter gene product or other selectable marker constructed so
25 that it will insert into the genome in operative association with the regulatory
element sequences of the native PNPG5 gene to be disrupted. Reporter genes that
can be used in practicing the invention are known in the art, and include those
encoding CAT, green fluorescent protein, and β -galactosidase, among others.
Nucleotide sequences encoding selectable markers are also known in the art, and

include those that encode gene products conferring resistance to antibiotics or anti-metabolites, or that supply an auxotrophic requirement.

[0208] In view of the present disclosure, methods that can be used for creating the genetic constructs of the present invention will be apparent, and can
5 include *in vitro* recombinant techniques, synthetic techniques, and *in vivo* genetic recombination, as described, among other places, in Ausubel *et al.*, 1989, above; Sambrook *et al.*, 1989, above; Innis *et al.*, 1995, above; and Erlich, 1992, above.

[0209] Mammalian cells can be transformed with a genetic construct of the present invention in accordance with known techniques, such as, *e.g.*, by
10 electroporation. Selection of transformants can be carried out using standard techniques, such as by selecting for cells expressing a selectable marker associated with the construct. Identification of transformants in which a successful recombination event has occurred and the particular target gene has been disabled can be carried out by genetic analysis, such as by Southern blot analysis, or by
15 Northern analysis to detect a lack of mRNA transcripts encoding the particular protein, or by the appearance of cells lacking the particular protein, as determined, *e.g.*, by immunological analysis, or some combination thereof.

[0210] The present invention thus provides modified mammalian cells in which the native PNPG5 gene has been mutated. The present invention further
20 provides modified animals in which the PNPG5 gene has been mutated.

5.8. Genetically Modified Animals

[0211] Genetically modified animals can be prepared for studying the biological function of PNPG5 *in vivo* and for screening and/or testing candidate compounds for their ability to affect the expression and/or activity of PNPG5 as
25 potential therapeutics for treating pain and related disorders such as chronic pain, neuropathic pain, inflammatory and cancer pain, addiction, seizure (including epilepsy), stroke or ischemia, neurodegenerative disorder (*e.g.*, Parkinson's disease, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), or Huntington's chorea), headache (*e.g.*, general, migraine, cluster or tension), anxiety, depression,

asthma, rheumatic disease, retinopathy, inflammatory eye disorders, pruritis, ulcer (gastric or duodenal), gastric lesions induced by a necrotizing agent, uncontrollable urination (*e.g.*, incontinence), inflammatory or unstable bladder disorders, or inflammatory bowel disease.

5 [0212] To investigate the function of PNPG5 *in vivo* in animals, PNPG5-encoding polynucleotides or PNPG5-inhibiting antisense nucleic acids, shRNAs, siRNAs, or ribozymes can be introduced into test animals, such as mice or rats, using, *e.g.*, viral vectors or naked nucleic acids. Alternatively, transgenic animals can be produced. Specifically, “knock-in” animals with the endogenous PNPG5
10 gene substituted with a heterologous gene or an ortholog from another species or a mutated PNPG5 gene, or “knockout” animals with PNPG5 gene partially or completely inactivated, or transgenic animals expressing or overexpressing a wild-type or mutated PNPG5 gene (*e.g.*, upon targeted or random integration into the genome) can be generated.

15 [0213] PNPG5-encoding nucleic acids can be introduced into animals using viral delivery systems. Exemplary viruses for production of delivery vectors include without limitation adenovirus, herpesvirus, retroviruses, vaccinia virus, and adeno-associated virus (AAV). See, *e.g.*, Becker *et al.*, *Meth. Cell Biol.* 1994; 43: 161-89; Douglas and Curiel, *Science & Medicine* 1997; 4: 44-53; Yeh and
20 Perricaudet, *FASEB J.* 1997; 11: 615-623; Kuo *et al.*, *Blood* 1993; 82: 845; Markowitz *et al.*, *J. Virol.* 1988; 62: 1120; Mann *et al.*, *Cell* 1983; 33: 153; U.S. Patents No. 5,399,346; 4,650,764; 4,980,289; 5,124,263; and International Publication No. WO 95/07358.

[0214] In an alternative method, a PNPG5-encoding nucleic acid can be
25 introduced by liposome-mediated transfection, a technique that provides certain practical advantages, including the molecular targeting of liposomes to specific cells. Directing transfection to particular cell types (also possible with viral vectors) is particularly advantageous in a tissue with cellular heterogeneity, such as the brain, pancreas, liver, and kidney. Lipids may be chemically coupled to other
30 molecules for the purpose of targeting. Targeted peptides (*e.g.*, hormones or

neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

[0215] In another embodiment, target cells can be removed from an animal, and a nucleic acid can be introduced as a naked construct. The transformed cells
5 can be then re-implanted into the body of the animal. Naked nucleic acid constructs can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter. See, *e.g.*, Wu et al., J. Biol. Chem. 1992; 267: 963-7; Wu et al., J.
10 Biol. Chem. 1988; 263: 14621-4.

[0216] In yet another embodiment, PNPG5-encoding nucleic acids can be introduced into animals by injecting naked plasmid DNA containing a PNPG5-encoding nucleic acid sequence into the tail vein of animals, in particular mammals (Zhang et al., Hum. Gen. Ther. 1999, 10:1735-7). This injection technique can
15 also be used to introduce siRNA targeted to PNPG5 into animals, in particular mammals (Lewis et al., *Nature Genetics* 2002, 32: 105-106).

[0217] As specified above, transgenic animals can also be generated. Methods of making transgenic animals are well-known in the art (for transgenic mice see *Gene Targeting: A Practical Approach*, 2nd Ed., Joyner ed., IRL Press at
20 Oxford University Press, New York, 2000; *Manipulating the Mouse Embryo: A Laboratory Manual*, Nagy et al. eds., Cold Spring Harbor Press, New York, 2003; *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson ed., IRL Press at Oxford University Press, 1987; *Transgenic Animal Technology: A Laboratory Handbook*, Pinkert ed., Academic Press, New York, 1994; Hogan,
25 *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1986; Brinster et al., *Proc. Nat. Acad. Sci. USA* 1985; 82: 4438- 4442; Capecchi, *Science* 1989; 244: 1288-1292; Joyner et al., *Nature* 1989; 338: 153-156; U.S. Patents No. 4,736,866; 4,870,009; 4,873,191; for particle bombardment see U.S. Patent No. 4, 945,050; for transgenic rats see, *e.g.*,
30 Hammer et al., *Cell* 1990; 63: 1099-1112; for non-rodent transgenic mammals and

other animals see, *e.g.*, Pursel *et al.*, *Science* 1989; 244: 1281-1288 and Simms *et al.*, *Bio/Technology* 1988; 6: 179- 183; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, *e.g.*, *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach*, Robertson ed., IRL Press, 1987). Clones of the nonhuman transgenic animals can be produced according to available methods (see *e.g.*, Wilmut *et al.*, *Nature* 1997; 385: 810-813 and International Publications No. WO 97/07668 and WO 97/07669).

10 [0218] In one embodiment, the transgenic animal is a "knockout" animal having a heterozygous or homozygous alteration in the sequence of an endogenous PNPG5 gene that results in a decrease of PNPG5 function, preferably such that PNPG5 expression is undetectable or insignificant. Knockout animals are typically generated by homologous recombination with a vector comprising a transgene
15 having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it.

[0219] Knockout animals can be prepared by any method known in the art (see, *e.g.*, Snouwaert *et al.*, *Science* 1992; 257: 1083; Lowell *et al.*, *Nature* 1993; 20 366: 740-42; Capecchi, *Science* 1989; 244: 1288-1292; Palmiter *et al.*, *Ann. Rev. Genet.* 1986; 20: 465-499; Bradley, *Current Opinion in Bio/Technology* 1991; 2: 823-829; and International Publications No. WO 90/11354, WO 91/01140, WO 92/0968, and WO 93/04169). Preparation of a knockout animal typically requires first introducing a nucleic acid construct (a "knockout construct"), that will be used
25 to decrease or eliminate expression of a particular gene, into an undifferentiated cell type termed an embryonic stem (ES) cell. The knockout construct is typically comprised of: (i) DNA from a portion (*e.g.*, an exon sequence, intron sequence, promoter sequence, or some combination thereof) of a gene to be knocked out; and (ii) a selectable marker sequence used to identify the presence of the knockout
30 construct in the ES cell. The knockout construct is typically introduced (*e.g.*, electroporated or microinjected) into ES cells so that it can homologously recombine

with the genomic DNA of the cell in a double crossover event. This recombined ES cell can be identified (*e.g.*, by Southern hybridization or PCR reactions that show the genomic alteration) and is then injected into a mammalian embryo at the blastocyst stage. In a preferred embodiment where the knockout animal is a mammal, a mammalian embryo with integrated ES cells is then implanted into a foster mother for the duration of gestation (see, *e.g.*, Zhou *et al.*, *Genes and Dev.* 1995; 9: 2623-34).

[0220] In a specific embodiment, the knockout vector is designed such that, upon homologous recombination, the endogenous PNPG5-related gene is functionally disrupted (*i.e.*, no longer encodes a functional protein). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous PNPG5-related gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the PNPG5-related polypeptide). In the homologous recombination vector, the altered portion of PNPG5-related gene is preferably flanked at its 5' and 3' ends by additional nucleic acid of the PNPG5-related gene to allow for homologous recombination to occur between the exogenous PNPG5-related gene carried by the vector and an endogenous PNPG5-related gene in an embryonic stem cell. The additional flanking PNPG5-related nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (at both the 5' and 3' ends) are included in the vector (see, *e.g.*, Thomas and Capecchi, *Cell* 1987; 51: 503). The vector is introduced into an ES cell line (*e.g.*, by electroporation), and cells in which the introduced PNPG5-related gene has homologously recombined with the endogenous PNPG5-related gene are selected (see, *e.g.*, Li *et al.*, *Cell* 1992; 69: 915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see, *e.g.*, Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson ed., IRL, Oxford, 1987, pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to

breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene.

[0221] The phenotype of knockout animals can be predictive of the *in vivo* function of the gene and of the effects or lack of effect of its antagonists or agonists.

5 Knockout animals can also be used to study the effects of the PNPG5 protein in models of disease, including, pain and pain-related disorders. In a specific embodiment, knockout animals, such as mice harboring the PNPG5 gene knockout, may be used to produce antibodies against the heterologous PNPG5 protein (*e.g.*, human PNPG5) (Claesson *et al.*, *Scan. J. Immunol.* 1994; 0: 257-264; Declerck *et*
10 *al.*, *J. Biol. Chem.* 1995; 270: 8397-400).

[0222] Genetically modified animals expressing or harboring PNPG5-specific antisense polynucleotides, shRNA, siRNA, or ribozymes can be used analogously to knockout animals described above.

[0223] In another embodiment of the invention, the transgenic animal is an
15 animal having an alteration in its genome that results in altered expression (*e.g.*, increased or decreased expression) of the PNPG5 gene, *e.g.*, by introduction of additional copies of PNPG5 gene in various parts of the genome, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the PNPG5 gene. Such regulatory sequences include inducible,
20 tissue-specific, and constitutive promoters and enhancer elements. Suitable promoters include metallothionein, albumin (Pinkert *et al.*, *Genes Dev.* 1987; 1: 268-76), and K-14 keratinocyte (Vassar *et al.*, *Proc. Natl. Acad. Sci. USA* 1989; 86: 1563-1567) gene promoters. Overexpression or underexpression of the wild-type PNPG5 polypeptide, polypeptide fragment or a mutated version thereof may
25 alter normal cellular processes, resulting in a phenotype that identifies a tissue in which PNPG5 expression is functionally relevant and may indicate a therapeutic target for the PNPG5, its agonists or antagonists. For example, a transgenic test animal can be engineered to overexpress or underexpress a full-length PNPG5
30 sequence, which may result in a phenotype that shows similarity with human diseases.

[0224] Transgenic animals can also be produced that allow for regulated (*e.g.*, tissue-specific) expression of the transgene. One example of such a system that may be produced is the Cre-Lox recombinase system of bacteriophage P1 (Lakso *et al.*, *Proc. Natl. Acad. Sci. USA* 1992; 89: 6232-6236; U.S. Patents No. 4,959,317 and 5,801,030). If the Cre-Lox recombinase system is used to regulate expression of a transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic or gene-targeted animals, one containing a transgene encoding a selected protein or containing a targeted allele (*e.g.*, a loxP flanked exon), and the other containing a transgene encoding a recombinase (*e.g.*, a tissue-specific expression of Cre recombinase). Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.*, *Science* 1991; 251: 1351-1355; U.S. Patent No. 5,654,182). In another embodiment, both Cre-Lox and Flp-Frt are used in the same system to regulate expression of the transgene, and for sequential deletion of vector sequences in the same cell (Sun *et al.*, *Nat. Genet.* 2000; 25: 83-6). Regulated transgenic animals can be also prepared using the tet-repressor system (see, *e.g.*, U.S. Patent No. 5,654,168).

[0225] The *in vivo* function of PNPG5 can be also investigated through making "knock-in" animals. In such animals the endogenous PNPG5 gene can be replaced, *e.g.*, by a heterologous gene, by a PNPG5 ortholog or by a mutated PNPG5 gene. See, for example, Wang *et al.*, *Development* 1997; 124: 2507-2513; Zhuang *et al.*, *Mol. Cell Biol.* 1998; 18: 3340-3349; Geng *et al.*, *Cell* 1999; 97: 767-777; Baudoin *et al.*, *Genes Dev.* 1998; 12: 1202-1216. Thus, a non-human transgenic animal can be created in which: (i) a human ortholog of the non-human animal PNPG5 gene has been stably inserted into the genome of the animal; and/or (ii) the endogenous non-human animal PNPG5 gene has been replaced with its human counterpart (see, *e.g.*, Coffman, *Semin. Nephrol.* 1997; 17: 404; Esther *et al.*, *Lab. Invest.* 1996; 74: 953; Murakami *et al.*, *Blood Press. Suppl.* 1996; 2: 36). In one aspect of this embodiment, a human PNPG5 gene inserted into the transgenic animal is the wild-type human PNPG5 gene. In another aspect, the PNPG5 gene

inserted into the transgenic animal is a mutated form or a variant of the human PNPG5 gene.

[0226] Included within the scope of the present invention are transgenic animals, preferably mammals (*e.g.*, mice) in which, in addition to the PNPG5 gene, one or more additional genes (preferably, associated with pain or related disorders) have been knocked out, or knocked in, or overexpressed. Such animals can be generated by repeating the procedures set forth herein for generating each construct, or by breeding two animals of the same species (each with a different single gene manipulated) to each other, and screening for those progeny animals having the desired genotype.

5.9. Use of the Nucleic Acid Molecules of the Invention to Modulate PNPG5 Gene Expression

[0227] As specified above, the PNPG5-encoding nucleic acid molecules of the invention or the nucleic acid molecules comprising sequences that hybridize to them under standard hybridization conditions (including their homologs/orthologs, complementary sequences and various oligonucleotide probes and primers derived from them) can be used to inhibit the expression of PNPG5 genes (*e.g.*, by inhibiting transcription, splicing, transport, or translation or by promoting degradation of corresponding mRNAs). Specifically, the nucleic acid molecules of the invention can be used to “knock down” or “knock out” the expression of the PNPG5 genes in a cell or tissue (*e.g.*, in an animal model or in cultured cells) by using their sequences to design antisense oligonucleotides, RNA interference (RNAi) molecules, ribozymes, nucleic acid molecules to be used in triplex helix formation, etc. Preferred methods to inhibit gene expression are described below.

5.9.1. RNA Interference (RNAi)

[0228] RNA interference (RNAi) is a process of sequence-specific post-transcriptional gene silencing by which double stranded RNA (dsRNA) homologous to a target locus can specifically inactivate gene function in plants, fungi, invertebrates, and vertebrates, including mammals (Hammond *et al.*, *Nature Genet.*

- 2001; 2: 110-119; Sharp, *Genes Dev.* 1999;13: 139-141). This dsRNA-induced gene silencing is mediated by short double-stranded small interfering RNAs (siRNAs) generated from longer dsRNAs by ribonuclease III cleavage (Bernstein *et al.*, *Nature* 2001; 409: 363-366 and Elbashir *et al.*, *Genes Dev.* 2001; 15: 188-200).
- 5 RNAi-mediated gene silencing is thought to occur via sequence-specific mRNA degradation, where sequence specificity is determined by the interaction of an siRNA with its complementary sequence within a target mRNA (see, *e.g.*, Tuschl, *Chem. Biochem.* 2001; 2: 239-245).

- [0229] For mammalian systems, RNAi commonly involves the use of dsRNAs that are greater than 500 bp; however, it can also be activated by
- 10 introduction of either siRNAs (Elbashir, *et al.*, *Nature* 2001; 411: 494-498) or short hairpin RNAs (shRNAs) bearing a fold back stem-loop structure (Paddison *et al.*, *Genes Dev.* 2002; 16: 948-958; Sui *et al.*, *Proc. Natl. Acad. Sci. USA* 2002; 99: 5515-5520; Brummelkamp *et al.*, *Science* 2002; 296: 550-553; Paul *et al.*, *Nature*
- 15 *Biotechnol.* 2002; 20: 505-508).

- [0230] The siRNAs to be used in the methods of the present invention are preferably short double stranded nucleic acid duplexes comprising annealed complementary single stranded nucleic acid molecules. Exemplary PNPG5-specific siRNA molecules (generated using Dharmacon siDESIGN program, Dharmacon,
- 20 Inc. available on the WorldWideWeb at design.dharmacon.com; see also Reynolds *et al.*, *Nature Biotechnol.*, 2004, 22: 326-330) are provided below:

SEQ ID NO:	Target sequence and corresponding antisense sequence	SEQ ID NO of target sequence source and Start of target sequence within source (nt)
46 47	Target:	SEQ ID NO: 1 (rat nt) 847
48 49	Target: T C A C G T G C G A T A C C A A A C A Antisense: T G T T T G G T A T C G C A C G T G A	SEQ ID NO: 1 (rat nt)

SEQ ID NO:	Target sequence and corresponding antisense sequence	SEQ ID NO of target sequence source and Start of target sequence within source (nt)
		863
50 51	Target: CTGTGGA ACTGATCAAGGA Antisense: TCCTTGATCAGTTCCACA G	SEQ ID NO: 3 (mouse nt) 521
52 53	Target: AGCAAGGCATGACCAAAGT Antisense: ACTTTGGTCATGCCTTGCT	SEQ ID NO: 3 (mouse nt) 881
54 55	Target: GCTCCGTGATGGACTTTCA Antisense: TGA AAGTCCATCACGGAG C	SEQ ID NO: 5 (human nt) 725
56 57	Target: GGCTGTACCTACATAACAA Antisense: TTGTTATGTAGGTACAGC C	SEQ ID NO: 5 (human nt) 644

[0231] In preferred embodiments, the siRNAs are short dsRNAs comprising annealed complementary single strand RNAs. However, the invention also encompasses embodiments in which the siRNAs comprise an annealed RNA:DNA duplex, wherein the sense strand of the duplex is a DNA molecule and the antisense strand of the duplex is a RNA molecule.

[0232] Preferably, each single stranded nucleic acid molecule of the siRNA duplex is of from about 19 nucleotides to about 27 nucleotides in length. In preferred embodiments, duplexed siRNAs have a 2 or 3 nucleotide 3' overhang on each strand of the duplex. In preferred embodiments, siRNAs have 5'-phosphate and 3'-hydroxyl groups.

[0233] The RNAi molecules to be used in the methods of the present invention comprise nucleic acid sequences that are complementary to the nucleic acid sequence of a portion of the target locus. In certain embodiments, the portion

of the target locus to which the RNAi probe is complementary is at least about 15 nucleotides in length. In preferred embodiments, the portion of the target locus to which the RNAi probe is complementary is at least about 19 nucleotides in length. The target locus to which an RNAi probe is complementary may represent a transcribed portion of the PNPG5 gene or an untranscribed portion of the PNPG5 gene (*e.g.*, intergenic regions, repeat elements, etc.).

[0234] The RNAi molecules may include one or more modifications, either to the phosphate-sugar backbone or to the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one heteroatom other than oxygen, such as nitrogen or sulfur. In this case, for example, the phosphodiester linkage may be replaced by a phosphothioester linkage. Similarly, bases may be modified to block the activity of adenosine deaminase. Where the RNAi molecule is produced synthetically, or by *in vitro* transcription, a modified ribonucleoside may be introduced during synthesis or transcription.

[0235] According to the present invention, siRNAs may be introduced to a target cell as an annealed duplex siRNA, or as single stranded sense and anti-sense nucleic acid sequences that, once within the target cell, anneal to form the siRNA duplex. Alternatively, the sense and anti-sense strands of the siRNA may be encoded on an expression construct that is introduced to the target cell. Upon expression within the target cell, the transcribed sense and antisense strands may anneal to reconstitute the siRNA.

[0236] The shRNAs to be used in the methods of the present invention comprise a single stranded "loop" region connecting complementary inverted repeat sequences that anneal to form a double stranded "stem" region. Structural considerations for shRNA design are discussed, for example, in McManus *et al.*, *RNA* 2002; 8: 842-850. In certain embodiments the shRNA may be a portion of a larger RNA molecule, *e.g.*, as part of a larger RNA that also contains U6 RNA sequences (Paul *et al.*, *supra*).

[0237] In preferred embodiments, the loop of the shRNA is from about 1 to about 9 nucleotides in length. In preferred embodiments the double stranded stem of the shRNA is from about 19 to about 33 base pairs in length. In preferred embodiments, the 3' end of the shRNA stem has a 3' overhang. In particularly preferred embodiments, the 3' overhang of the shRNA stem is from 1 to about 4 nucleotides in length. In preferred embodiments, shRNAs have 5'-phosphate and 3'-hydroxyl groups.

[0238] Although the RNAi molecules useful according to the invention preferably contain nucleotide sequences that are fully complementary to a portion of the target locus, 100% sequence complementarity between the RNAi probe and the target locus is not required to practice the invention.

[0239] RNA molecules useful for RNAi may be chemically synthesized, for example using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. RNAs produced by such methodologies tend to be highly pure and to anneal efficiently to form siRNA duplexes or shRNA hairpin stem-loop structures. Following chemical synthesis, single stranded RNA molecules are deprotected, annealed to form siRNAs or shRNAs, and purified (*e.g.*, by gel electrophoresis or HPLC).

[0240] Alternatively, standard procedures may used for *in vitro* transcription of RNA from DNA templates carrying RNA polymerase promoter sequences (*e.g.*, T7 or SP6 RNA polymerase promoter sequences). Efficient *in vitro* protocols for preparation of siRNAs using T7 RNA polymerase have been described (Donzé and Picard, *Nucleic Acids Res.* 2002; 30: e46; and Yu *et al.*, *Proc. Natl. Acad. Sci. USA* 2002; 99: 6047-6052). Similarly, an efficient *in vitro* protocol for preparation of shRNAs using T7 RNA polymerase has been described (Yu *et al.*, *supra*). The sense and antisense transcripts may be synthesized in two independent reactions and annealed later, or may be synthesized simultaneously in a single reaction.

[0241] RNAi molecules may be formed within a cell by transcription of RNA from an expression construct introduced into the cell. For example, both a

protocol and an expression construct for *in vivo* expression of siRNAs are described in Yu *et al.*, *supra*. Similarly, protocols and expression constructs for *in vivo* expression of shRNAs have been described (Brummelkamp *et al.*, *supra*; Sui *et al.*, *supra*; Yu *et al.*, *supra*; McManus *et al.*, *supra*; Paul *et al.*, *supra*).

5 [0242] The expression constructs for *in vivo* production of RNAi molecules comprise RNAi encoding sequences operably linked to elements necessary for the proper transcription of the RNAi encoding sequence(s), including promoter elements and transcription termination signals. Preferred promoters for use in such expression constructs include the polymerase-III HI-RNA promoter (see, *e.g.*,
10 Brummelkamp *et al.*, *supra*) and the U6 polymerase-III promoter (see, *e.g.*, Sui *et al.*, *supra*; Paul, *et al.* *supra*; and Yu *et al.*, *supra*). The RNAi expression constructs can further comprise vector sequences that facilitate the cloning of the expression constructs. Standard vectors that may be used in practicing the current invention are known in the art (*e.g.*, pSilencer 2.0-U6 vector, Ambion Inc., Austin,
15 TX).

5.9.2. Antisense Nucleic Acids

[0243] In a specific embodiment, to achieve inhibition of expression of a PNPG5 gene, the nucleic acid molecules of the invention can be used to design antisense oligonucleotides. An antisense oligonucleotide is typically 18 to 25 bases
20 in length (but can be as short as 13 bases in length) and is designed to bind to a selected PNPG5 mRNA. This binding prevents expression of that specific PNPG5 protein. The antisense oligonucleotides of the invention comprise at least 6 nucleotides and preferably comprise from 6 to about 50 nucleotides. In specific aspects, the antisense oligonucleotides comprise at least 10 nucleotides, at least 15
25 nucleotides, at least 25, at least 30, at least 100 nucleotides, or at least 200 nucleotides.

[0244] The antisense nucleic acid oligonucleotides of the invention comprise sequences complementary to at least a portion of the corresponding PNPG5 mRNA. However, 100% sequence complementarity is not required so long as formation of a

stable duplex (for single stranded antisense oligonucleotides) or triplex (for double stranded antisense oligonucleotides) can be achieved. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense oligonucleotides. Generally, the longer the antisense oligonucleotide, the more base mismatches with the corresponding mRNA can be tolerated. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

[0245] The antisense oligonucleotides can be DNA or RNA or chimeric mixtures, or derivatives or modified versions thereof, and can be single-stranded or double-stranded. The antisense oligonucleotides can be modified at the base moiety, sugar moiety, or phosphate backbone, or a combination thereof. For example, a PNPG5-specific antisense oligonucleotide can comprise at least one modified base moiety selected from a group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

[0246] In another embodiment, the PNPG5-specific antisense oligonucleotide comprises at least one modified sugar moiety, *e.g.*, a sugar moiety selected from arabinose, 2-fluoroarabinose, xylulose, and hexose.

[0247] In yet another embodiment, the PNPG5-specific antisense oligonucleotide comprises at least one modified phosphate backbone selected from a

phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

[0248] The antisense oligonucleotide can include other appending groups
5 such as peptides, or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* 1989; 86: 6553-6556; Lemaitre *et al.*, *Proc. Natl. Acad. Sci. USA* 1987; 84: 648-652; PCT Publication No. WO 88/09810) or blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134), hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.*, *BioTechniques* 1988;
10 6: 958-976), intercalating agents (see, *e.g.*, Zon, *Pharm. Res.* 1988; 5: 539-549), etc.

[0249] In another embodiment, the antisense oligonucleotide can include α -anomeric oligonucleotides. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units,
15 the strands run parallel to each other (Gautier *et al.*, *Nucl. Acids Res.* 1987; 15: 6625-6641).

[0250] In yet another embodiment, the antisense oligonucleotide can be a morpholino antisense oligonucleotide (*i.e.*, an oligonucleotide in which the bases are linked to 6-membered morpholine rings, which are connected to other morpholine-linked bases via non-ionic phosphorodiamidate intersubunit linkages). Morpholino
20 oligonucleotides are resistant to nucleases and act by sterically blocking transcription of the target mRNA.

[0251] Similar to the above-described RNAi molecules, the antisense oligonucleotides of the invention can be synthesized by standard methods known in
25 the art, *e.g.*, by use of an automated synthesizer. Antisense nucleic acid oligonucleotides of the invention can also be produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell within which the vector or a portion thereof is transcribed to produce an antisense RNA. Such a vector can remain episomal or

become chromosomally integrated, so long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. In
5 another embodiment, “naked” antisense nucleic acids can be delivered to adherent cells via “scrape delivery”, whereby the antisense oligonucleotide is added to a culture of adherent cells in a culture vessel, the cells are scraped from the walls of the culture vessel, and the scraped cells are transferred to another plate where they are allowed to re-adhere. Scraping the cells from the culture vessel walls serves to
10 pull adhesion plaques from the cell membrane, generating small holes that allow the antisense oligonucleotides to enter the cytosol.

[0252] The present invention thus provides a method for inhibiting the expression of a PNPG5 gene in a eukaryotic, preferably mammalian, and more preferably rat, mouse or human, cell, comprising providing the cell with an
15 effective amount of a PNPG5-inhibiting antisense oligonucleotide.

5.9.3. Ribozyme Inhibition

[0253] In another embodiment, the expression of PNPG5 genes of the present invention can be inhibited by ribozymes designed based on the nucleotide sequence thereof.

20 [0254] Ribozyme molecules catalytically cleave mRNA transcripts and can be used to prevent expression of the gene product. Ribozymes are enzymatic RNA molecules capable of catalyzing the sequence-specific cleavage of RNA (for a review, see Rossi, *Current Biology* 1994; 4: 469-471). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to
25 complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include: (i) one or more sequences complementary to the target gene mRNA; and (ii) a catalytic sequence responsible for mRNA cleavage (see, *e.g.*, U.S. Patent No. 5,093,246).

[0255] According to the present invention, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA has the following sequence
5 of two bases: 5'-UG-3'. The construction of hammerhead ribozymes is known in the art, and described more fully in Myers, *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, New York, 1995 (see especially Figure 4, page 833) and in Haseloff and Gerlach, *Nature* 1988; 334: 585-591.

[0256] Preferably, the ribozymes of the present invention are engineered so
10 that the cleavage recognition site is located near the 5' end of the corresponding mRNA, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

[0257] As in the case of RNAi and antisense oligonucleotides, ribozymes of the invention can be composed of modified oligonucleotides (*e.g.*, for improved
15 stability, targeting, etc.). These can be delivered to mammalian cells, and preferably mouse, rat, or human cells, which express the target PNPG5 protein *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy
20 endogenous mRNA encoding the protein and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration may be required to achieve an adequate level of efficacy.

[0258] Ribozymes can be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. Ribozyme technology
25 is described further in *Intracellular Ribozyme Applications: Principals and Protocols*, Rossi and Couture eds., Horizon Scientific Press, 1999.

5.9.4. Triple Helix Formation

[0259] Nucleic acid molecules useful to inhibit PNPG5 gene expression via triple helix formation are preferably composed of deoxynucleotides. The base

composition of these oligonucleotides is typically designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, resulting in TAT and CGC triplets
5 across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, *e.g.*, those containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in
10 GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

[0260] Alternatively, sequences can be targeted for triple helix formation by creating a so-called “switchback” nucleic acid molecule. Switchback molecules are
15 synthesized in an alternating 5’-3’, 3’-5’ manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

[0261] Similarly to PNPg5-specific RNAi, antisense oligonucleotides, and ribozymes, triple helix molecules of the invention can be prepared by any method
20 known in the art. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides such as, *e.g.*, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules can be generated by *in vitro* or *in vivo* transcription of DNA sequences “encoding” the particular RNA molecule. Such DNA sequences can be incorporated into a wide
25 variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters.

5.10. Diagnostic Methods

[0262] A variety of methods can be employed for the diagnostic evaluation of pain, and for the identification and evaluation of subjects experiencing pain and

related disorders. These methods may utilize reagents such as the polynucleotide molecules and oligonucleotides of the present invention. The methods may alternatively utilize a PNPG5 protein or a fragment thereof, or an antibody or antibody fragment that binds specifically to a PNPG5 protein. Such reagents can be used for: (i) the detection of either an over- or an under-expression of the PNPG5 gene relative to its expression in an unaffected state (*e.g.*, in a subject or individual not having pain or a related disorder); or (ii) the detection of either an increase or a decrease in the level of the PNPG5 protein relative to its level in an unaffected state; or (iii) the detection of an aberrant PNPG5 gene product activity relative to the unaffected state.

[0263] In a preferred embodiment, a diagnostic method of the present invention utilizes quantitative hybridization (*e.g.*, quantitative *in situ* hybridization, Northern blot analysis or microarray hybridization) or quantitative PCR (*e.g.*, TaqMan®) using a PNPG5-specific nucleic acid of the invention as a hybridization probe and PCR primers, respectively.

[0264] The present invention provides a method for detecting a pain response in a test cell subjected to a treatment or stimulus or suspected of having been subjected to a treatment or stimulus, said method comprising:

- (a) determining the expression level in the test cell of a nucleic acid molecule encoding a PNPG5 protein; and
- (b) comparing the expression level of the PNPG5-encoding nucleic acid molecule in the test cell to the expression level of the same nucleic acid molecule in a control cell not subjected to a treatment or stimulus;

wherein a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule in the test cell compared to the expression level of the PNPG5-encoding nucleic acid molecule in the control cell indicates that the test cell is exhibiting a pain response.

[0265] According to the present invention, the detectable change in the expression level is any statistically significant change and preferably at least a 1.5-fold change as measured by any available technique such as hybridization or quantitative PCR (*see* the Definitions Section, above).

5 [0266] The present invention further provides a method for detecting a pain response in a test cell, said method comprising:

- (a) determining in a test cell capable of expressing a PNPG5 protein the expression level of the protein; and
- (b) comparing the expression level of the PNPG5 protein in the
10 test cell to the expression level of the PNPG5 protein in a control cell not exhibiting a pain response;

wherein a detectable difference between the expression level of the PNPG5 protein in the test cell and the expression level of the PNPG5 protein in the control cell indicates that the test cell is exhibiting a pain response. According to the present
15 invention, the detectable change in the expression level of a PNPG5 protein is any statistically significant change and preferably at least a 1.5-fold change as measured by any available technique such as protein staining or immunodetection.

[0267] The present invention further provides a method for detecting a pain response in a test cell, said method comprising:

- 20 (a) determining an activity level in the test cell of a PNPG5 protein; and
- (b) comparing the activity level of the PNPG5 protein in the test cell to the activity level of the PNPG5 protein in a control cell not exhibiting a pain response;

25 wherein a detectable difference between the activity level of the PNPG5 protein in the test cell and the activity level of the PNPG5 protein in the control cell indicates that the test cell is exhibiting a pain response.

[0268] The test and control cells are preferably the same type of cells from the same species and tissue, and can be any cells useful for conducting this type of assay where a meaningful result can be obtained. Any cell type in which a PNPG5-encoding polynucleotide molecule is ordinarily expressed, or in which a PNPG5-
5 encoding polynucleotide is expressed, or in which a PNPG5 protein is expressed, or in which an activity of a PNPG5 protein changes (as appropriate to the particular method) in connection with pain or a related treatment or stimulus, may be used. For example, the test cell can be any cell derived from a tissue of an organism experiencing a feeling of pain or associated disorder. Alternatively, the test cell can
10 be any cell grown *in vitro* under specific conditions. When the test cell is derived from a tissue of an organism experiencing a feeling of pain or associated disorder, it may or may not be known to be located in the region associated with the feeling of pain.

[0269] In one embodiment, the test and control cells are cells from the
15 central nervous system (CNS) or peripheral nervous system (PNS). Preferably, the test and control cells are neuronal cells from the dorsal root ganglia (DRG). The test and control cells can be derived from any appropriate organism, but are preferably human, rat or mouse cells. In a specific embodiment, the test and control cells are from an animal model of pain (*e.g.*, rat SNL model of neuropathic
20 pain) or any related disorder, and may or may not be isolated from that animal model. In another embodiment, the first cell is from a subject, such as a human or companion animal, for which the test is being conducted to determine the pain state of that subject, and the second cell is an appropriate control cell. The first cell may or may not be isolated from the subject being tested. Both the test cell and the
25 control cell must have the ability to express PNPG5.

[0270] The control cell can be any cell which is known to have not been subjected to any treatment or stimulus associated with pain. Preferably, the control cell is otherwise similar and treated identically to the test cell. For example, when the test cell is derived from a tissue of an animal experiencing a feeling of pain or
30 associated disorder, the control cell can be derived from an identical tissue or body part of a different animal from, preferably, the same species (or, alternatively, a

closely related species) which animal is not experiencing a feeling of pain or associated disorder. Alternatively, the control cell can be derived from an identical tissue or body part of the same animal from which the test cells are derived. However if this is the case, it should be established that the identical tissue or body
5 part has not been subjected to any treatment or stimulus associated with pain within the timeframe of the experiment. When the test cell is a cell grown *in vitro* under specific conditions, the control cell can be a similar cell grown *in vitro* in identical conditions but in the absence of the treatment or stimulus.

[0271] In one embodiment, the test cell has been exposed to a treatment or
10 stimulus that is, or that simulates or mimics, a pain condition prior to comparing the expression level of the polynucleotide molecule encoding the PNPG5 protein, or the expression level of the PNPG5 protein, or an activity of the PNPG5 protein (as appropriate to the particular method), with that of a control cell useful as an appropriate comparator cell to allow a determination of whether or not the test cell
15 is exhibiting a pain response. For example, where the test cell has been exposed to a treatment or stimulus that is, or that simulates or mimics, a pain condition, the control cell has not been exposed to such a treatment or stimulus. In another embodiment, the test cell has been exposed to a compound that is being tested to determine whether it simulates or mimics a pain condition.

20 [0272] In one embodiment, the nucleic acid molecule the expression of which is being determined according to this method encodes a mammalian PNPG5 protein. In one embodiment, the nucleic acid molecule encodes a rat, mouse or human PNPG5 protein. In one embodiment, the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2. In another embodiment, the mouse PNPG5
25 protein comprises the amino acid sequence of SEQ ID NO:4. In another embodiment, the human PNPG5 protein comprises the amino acid sequence of SEQ ID NO:6.

[0273] In one embodiment, the expression level of the nucleic acid molecule in each of the test and control cells is determined by quantifying the amount of
30 PNPG5-encoding mRNA present in the two cells. In another embodiment, the

expression level of the nucleic acid molecule in each of the test and control cells is determined by quantifying the amount of PNPG5 protein present in each of the two cells. Where the test cell has a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule compared to the expression level of the PNPG5-encoding nucleic acid molecule in the control cell, a pain response in the test cell has been detected.

[0274] To assay levels of a PNPG5-encoding nucleic acid in a sample, a variety of standard nucleic acid isolation and quantification methods can be employed. As specified above, in a preferred embodiment, a diagnostic method of the present invention utilizes quantitative hybridization (*e.g.*, quantitative *in situ* hybridization, Northern blot analysis or microarray hybridization) or quantitative PCR (*e.g.*, TaqMan®) using PNPG5-specific nucleic acids of the invention as hybridization probes and PCR primers, respectively.

[0275] In PCR-based assays, gene expression can be measured after extraction of cellular mRNA and preparation of cDNA by reverse transcription (RT). A sequence within the cDNA can then be used as a template for a nucleic acid amplification reaction. Nucleic acid molecules of the present invention can be used to design PNPG5-specific RT and PCR oligonucleotide primers (such as, *e.g.*, SEQ ID NOS: 14, 15, 17, 18, and 23-29, see **Table 2**, below). Preferably, the oligonucleotide primers are at least about 9 to about 30 nucleotides in length. The amplification can be performed using, *e.g.*, radioactively labeled or fluorescently-labeled nucleotides, for detection. Alternatively, enough amplified product may be made such that the product can be visualized simply by standard ethidium bromide or other staining methods.

[0276] A preferred PCR-based detection method of the present invention is quantitative real time PCR (*e.g.*, TaqMan® technology, Applied Biosystems, Foster City, CA). This method is based on the observation that there is a quantitative relationship between the amount of the starting target molecule and the amount of PCR product produced at any given cycle number. Real time PCR detects the accumulation of amplified product during the reaction by detecting a fluorescent

signal produced proportionally during the amplification of a PCR product. The method takes advantage of the properties of Taq DNA polymerases having 5' exonuclease activity (*e.g.*, AmpliTaq®) and Fluorescent Resonant Energy Transfer (FRET) method for detection in real time. The 5' exonuclease activity of the Taq DNA polymerase acts upon the surface of the template to remove obstacles downstream of the growing amplicon that may interfere with its generation. FRET is based on the observation that when a high-energy dye is in close proximity to a low-energy dye, a transfer of energy from high to low will typically occur. The real time PCR probe is designed with a high-energy dye termed a "reporter" at the 5' end, and a low-energy molecule termed a "quencher" at the 3' end. When this probe is intact and excited by a light source, the reporter dye's emission is suppressed by the quencher dye as a result of the close proximity of the dyes. When the probe is cleaved by the 5' nuclease activity of the Taq enzyme, the distance between the reporter and the quencher increases, causing the transfer of energy to stop, resulting in an increase of fluorescent emissions of the reporter, and a decrease in the fluorescent emissions of the quencher. The increase in reporter signal is captured by the Sequence Detection instrument and displayed. The amount of reporter signal increase is proportional to the amount of product being produced for a given sample. According to this method, the data is preferably measured at the exponential phase of the PCR reaction.

[0277] Specifically, a fluorogenic probe complementary to the target sequence is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome (*e.g.*, 6-carboxyfluorescein (6-FAM)). A quencher fluorochrome (*e.g.*, 6-carboxy-tetramethyl-rhodamine (TAMRA)) is added at any T position or at the 3' end. The probe is designed to have a higher melting temperature (T_m) than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as Taq polymerase extends the primer, the intrinsic 5' nuclease activity of Taq degrades the probe, releasing the reporter fluorochrome which results in an increase in the fluorescence

intensity of the reporter dye. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. This process occurs in every cycle and does not interfere with the accumulation of PCR product.

5 [0278] In a high throughput setting, to induce fluorescence during PCR, laser light is distributed to 96 sample wells via a multiplexed array of optical fibers. The resulting fluorescent emission returns via the fibers and is directed to a spectrograph with a charge-coupled device (CCD) camera. Emissions sent through the fiber to the CCD camera are analyzed by the software's algorithms. Collected
10 data are subsequently sent to the computer. Emissions are measured, *e.g.*, every 7 seconds. The sensitivity of detection allows acquisition of data when PCR amplification is still in the exponential phase and makes real time PCR more reliable than end-point measurements of accumulated PCR products used by traditional PCR methods.

15 [0279] Some of the preferred parameters of the quantitative real time PCR reactions of the present invention include: (i) designing the probe so that its T_m is 10°C higher than for the PCR primers, (ii) having primer T_m 's between 58°C and 60°C, (iii) having amplicon sizes between 50 and 150 bases, and (iv) avoiding 5' Gs. However, other parameters can be used (*e.g.*, determined using Primer
20 Express® software, Applied Biosystems, Foster City, CA). For example, the best design for primers and probes to use for the quantitation of mRNA expression involves positioning of a primer or probe over an intron.

[0280] For more details on quantitative real time PCR, see Gibson *et al.*, *Genome Res.* 1996; 6: 995-1001; Heid *et al.*, *Genome Res.* 1996; 6: 986-994; Livak
25 *et al.*, *PCR Methods Appl.* 1995; 4: 357-362; Holland *et al.*, *Proc. Natl. Acad. Sci. USA* 1991; 88: 7276-7280.

[0281] SYBR Green Dye PCR (Molecular Probes, Inc., Eugene, OR), competitive PCR as well as other quantitative PCR techniques can also be used to quantify PNPG5 gene expression according to the present invention.

[0282] PNP5 gene expression detection assays of the invention can also be performed *in situ* (e.g., directly upon sections of fixed or frozen tissue collected from a subject, thereby eliminating the need for nucleic acid purification). Nucleic acid molecules of the invention or portions thereof can be used as labeled probes or
5 primers for such *in situ* procedures (see, e.g., **Figure 5** and Section 6.5., below; see also, e.g., Nuovo, *PCR in situ Hybridization: Protocols And Application*, Raven Press, New York, 1992). Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard quantitative Northern analysis can be performed to determine the level of gene expression using the nucleic acid molecules of the
10 invention or portions thereof as labeled probes (see, e.g., **Figure 2** and section 6.4., below).

[0283] For *in vitro* cell cultures or *in vivo* animal models, the diagnostic reagents of the invention can be used in screening assays as surrogates for the pain state to find compounds that affect expression of the PNP5 gene. For example,
15 probes for the human PNP5 gene can be used for diagnosing individuals experiencing a pain or a related condition, and also for monitoring the effectiveness of a pain therapy.

[0284] Various techniques can be used to measure the levels of PNP5 protein in a sample, including the use of anti-PNP5 antibodies or antibody
20 fragments. Antibodies and various immunoassay methods also have important applications for assessing the efficacy of pain treatments. For example, anti-PNP5 antibodies or antibody fragments can be used to screen test compounds to identify those compounds that can modulate PNP5 protein production. For example, anti-PNP5 antibodies or antibody fragments can be used to detect the presence of the
25 PNP5 protein by, e.g., immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric or fluorimetric detection methods. Such techniques are particularly preferred for detecting the presence of the PNP5 protein on the surface of cells. In addition, protein isolation methods such as those described by Harlow and Lane (*Antibodies: A Laboratory*
30 *Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988) can also be employed to measure the levels of PNP5 protein in a sample.

- [0285] Antibodies or antigen-binding fragments thereof may also be employed histologically, *e.g.*, in immunofluorescence or immunoelectron microscopy techniques, for *in situ* detection of the PNPG5 protein. *In situ* detection may be accomplished by, *e.g.*, removing a tissue sample from a patient and
5 applying to the tissue sample a labeled antibody or antibody fragment of the present invention. This procedure can be used to detect both the presence of the PNPG5 protein and its distribution in the tissue. Additionally, antibodies or antigen-binding fragments may be used to detect PNPG5 protein in the serum of cells, tissues, or animals that produced PNPG5 protein.
- 10 [0286] Assays to determine the activity of PNPG5 are discussed, *infra*. In a specific embodiment, the activity of the PNPG5 protein is its ability to interact with (*e.g.*, bind to and/or co-immunoprecipitate with) a protein comprising an Leucine Rich Repeat (LRR) domain such as, for example, a PNPG2, PNPG5, PNPG3/AMIGO3, or AMIGO2 protein. In another specific embodiment, the
15 activity of the PNPG5 protein is the stimulation of neurite extension of hippocampal neurons (Kuja-Panula *et al.*, *J. Cell Biol.*, 160:963-973 (2003)). In yet another specific embodiment, the activity of the PNPG5 protein is the ability to promote PNPG2-mediated signaling.

5.11. Screening Methods

- 20 [0287] The present invention further provides a method for identifying a candidate compound useful for modulating the expression of a PNPG5-encoding nucleic acid, said method comprising:
- (a) contacting a first cell with a test compound under conditions sufficient to allow the cell to respond to said contact with the test
25 compound;
 - (b) determining the expression level of a PNPG5-encoding nucleic acid molecule in the cell prepared in step (a); and
 - (c) comparing the expression level of the PNPG5-encoding nucleic acid molecule determined in step (b) to the expression level of the

PNPG5-encoding nucleic acid molecule in a second (control) cell that has not been contacted with the test compound;

wherein a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule in the first cell in response to contact with the test compound
5 compared to the expression level of the PNPG5-encoding nucleic acid molecule in the second (control) cell that has not been contacted with the test compound, indicates that the test compound modulates the expression of the PNPG5-encoding nucleic acid.

[0288] In one embodiment, the candidate compound decreases the expression
10 of the PNPG5-encoding nucleic acid molecule. In another embodiment, the candidate compound increases the expression of the PNPG5-encoding nucleic acid molecule. In another embodiment, the first and second cells are incubated under conditions that induce the expression of a PNPG5-encoding nucleic acid molecule, but the test compound is tested for its ability to inhibit or reduce the induction of
15 such expression in the first cell. In another embodiment, the first and second cells are incubated under conditions that induce the expression of a PNPG5-encoding nucleic acid molecule, but the test compound is tested for its ability to potentiate the induction of such expression in the first cell.

[0289] This method of the present invention can be used to identify a
20 candidate compound useful to treat a condition that can be treated by modulating the expression of a PNPG5-encoding nucleic acid of the present invention.

[0290] The test compound can be, without limitation, a small organic or inorganic molecule, a polypeptide (including an antibody, antibody fragment, or other immunospecific molecule), an oligonucleotide molecule, a polynucleotide
25 molecule, or a chimera or derivative thereof.

[0291] The first and second cells are preferably the same types of cells, and can be any cells useful for conducting this type of assay where a meaningful result can be obtained. Such cells can be prokaryotic, but are preferably eukaryotic. Such

eukaryotic cells are preferably mammalian cells, and more preferably rat, mouse or human cells. Both the first and second cell must have the ability to express PNPG5. In one non-limiting embodiment, the first and second cells are cells that have been genetically modified to express or over-express a PNPG5 nucleic acid molecule. In
5 another non-limiting embodiment, the first and second cells are cells that express a PNPG5 nucleic acid molecule, either naturally (*e.g.*, DRG cells) or in response to an appropriate stimulus. In one embodiment, the first and second cells have been exposed to a condition or stimulus that is, or that simulates or mimics, a pain condition prior to, or at the same time as, exposing the cells to the test compound to
10 determine the effect of the test compound on the expression level of the nucleic acid molecule encoding the PNPG5 protein.

[0292] In one embodiment, the first and second cells are from an animal model of pain (*e.g.*, rat SNL model of neuropathic pain or other animal models described below), and may or may not be isolated from that animal model. In
15 another embodiment, the first cell is from a subject, such as a human or companion animal, and the second cell is an appropriate control cell. The first cell may or may not be isolated from the subject being tested.

[0293] In one embodiment, the nucleic acid molecule the expression of which is being determined according to this method encodes a mammalian PNPG5
20 protein. In a further embodiment, the nucleic acid molecule encodes a rat, mouse or human PNPG5 protein. In one embodiment, the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2. In another embodiment, the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO:4. In another embodiment, the human PNPG5 protein comprises the amino acid sequence of SEQ
25 ID NO:6.

[0294] The expression level of the nucleic acid molecule in each of the first and second cells can be determined by quantifying and comparing the amount of PNPG5-encoding mRNA present in each of the first and second cells. Alternatively, the expression level of the nucleic acid molecule in each of the first
30 and second cells can be determined by quantifying and comparing the amount of

PNPG5 protein present in the first and second cells. Where the first cell has a detectable change in the expression level of the nucleic acid encoding a PNPG5 protein compared to the expression level of the nucleic acid encoding the PNPG5 protein in the second cell, the test compound is identified as a candidate compound
5 useful for modulating the expression of a PNPG5-encoding nucleic acid.

[0295] In one non-limiting embodiment, ordered differential display (ODD, Matz *et al.*, *Nucleic Acid Res.* 1997, 25: 2541-2542) can be used to identify and evaluate compounds for their ability to modulate the expression of PNPG5-encoding nucleic acids in a similar manner to Kuja-Panula *et al.* (*J. Cell Biol.* 2003, 160:
10 963-973) who used ODD to determine that amphoterin and RAGE (receptor for advanced glycosylation end products) induce the expression of PNPG5 nucleic acid.

[0296] The present invention further provides a method for monitoring the efficacy of an analgesic treatment in a cell comprising:

- 15 (a) administering to said cell an analgesic compound under conditions sufficient to allow the cell to respond to said compound;
- (b) determining the expression level of a PNPG5-encoding nucleic acid molecule in the cell prepared in step (a); and
- 20 (c) comparing the expression level of the PNPG5-encoding nucleic acid molecule determined in step (b) to the expression level of the PNPG5-encoding nucleic acid molecule in a second (control) cell that has not been contacted with the analgesic compound;

wherein a detectable change in the expression level of the PNPG5-encoding nucleic
25 acid molecule in the first cell in response to contact with the analgesic compound compared to the expression level of the PNPG5-encoding nucleic acid molecule in the second (control) cell that has not been contacted with the analgesic compound is indicative of the activity of the analgesic compound.

[0297] Also provided herein is a method for identifying a candidate compound useful for modulating the expression of a PNPG5 protein, said method comprising:

- 5 (a) contacting a first cell with a test compound under conditions sufficient to allow the cell to respond to said contact with the test compound;
- (b) determining the expression level of a PNPG5 protein in the cell prepared in step (a); and
- 10 (c) comparing the expression level of the PNPG5 protein determined in step (b) to the expression level of the PNPG5 protein in a second (control) cell that has not been contacted with the test compound;

wherein a detectable change in the expression level of the PNPG5 protein in the first cell in response to contact with the test compound compared to the expression level
15 of the PNPG5 protein in the second (control) cell that has not been contacted with the test compound, indicates that the test compound modulates the expression of the PNPG5 protein and is a candidate compound.

[0298] The present invention further provides a method for identifying a compound useful for modulating an activity of a PNPG5 protein, said method
20 comprising:

- (a) contacting a PNPG5 protein with a test compound under conditions sufficient to allow the protein to respond to said contact with the test compound;
- 25 (b) determining the level of an activity of the PNPG5 protein during or after contact with the test compound; and

- (c) comparing the activity of the PNPG5 protein determined in step (b) to the activity of the PNPG5 protein when it is not in contact with the test compound;

wherein a detectable difference between the activity of the PNPG5 protein in response to contact with the test compound and the activity of the PNPG5 protein when it is not in contact with the test compound indicates that the test compound modulates the activity of the PNPG5 protein. A test compound that can modulate an activity of the PNPG5 protein may be considered to be a candidate compound, which can be further tested and characterized.

[0299] PNPG5 activity can include a specific binding of PNPG5 to a binding partner and/or the propagation of a transmembrane signal (*e.g.*, stimulation of a G protein such as PNPG2). In a specific embodiment, the activity of the PNPG5 protein is its ability to interact with (*e.g.*, bind to and/or co-immunoprecipitate with) a protein comprising an Leucine Rich Repeat (LRR) domain such as, for example, a PNPG2, PNPG5, PNPG3/AMIGO3, or AMIGO2 protein. In another specific embodiment, the activity of the PNPG5 protein is the stimulation of neurite extension of hippocampal neurons (Kuja-Panula *et al.*, *J. Cell Biol.*, 160:963-973 (2003)). In yet another specific embodiment, the activity of the PNPG5 protein is the ability to promote PNPG2-mediated signaling.

[0300] The present invention further provides a method for identifying a candidate compound capable of binding to a PNPG5 protein, said method comprising:

- (a) contacting a PNPG5 protein with a test compound under conditions that permit binding of the test compound to the PNPG5 protein; and
- (b) detecting binding of the test compound to the PNPG5 protein.

[0301] In one embodiment of the above method, the test compound is a protein comprising an LRR domain. In another embodiment of the above method,

the test compound is a PNPG2 protein. In another embodiment, the test compound is not a PNPG5 protein; an AMIGO2 protein; or a PNPG3/AMIGO3 protein.

[0302] The present invention further provides a method for identifying a compound capable of modulating binding between a PNPG5 protein and a PNPG2
5 protein, said method comprising determining the ability of the PNPG5 protein and the PNPG2 protein to bind together in the presence and absence of a test compound.

[0303] In one embodiment, the method comprises:

- 10 (a) combining a PNPG5 protein and a PNPG2 protein in the presence of a test compound under conditions that would permit binding of the PNPG5 protein to the PNPG2 protein in the absence of the test compound;
- (b) determining the degree of binding of the PNPG5 protein to the PNPG2 protein in the presence of the test compound; and
- 15 (c) comparing the degree of binding of the PNPG5 protein to the PNPG2 protein in the presence of the test compound with the degree of binding of the PNPG5 protein to the PNPG2 protein in the absence of the test compound;

wherein a detectable difference between the amount of the PNPG5 protein binding to the PNPG2 protein in the presence of the test compound and the amount of the PNPG5 protein binding to the PNPG2 protein in the absence of the test compound
20 indicates that the test compound modulates binding between the PNPG5 protein and the PNPG2 protein.

[0304] In one embodiment, the PNPG5 protein and the PNPG2 protein are both soluble proteins. In another embodiment, the PNPG5 protein is a soluble protein and the PNPG2 protein is present in a membrane fraction prepared from a
25 cell. In another embodiment, the PNPG2 protein is a soluble protein and the PNPG5 protein is present in a membrane fraction prepared from a cell. In another embodiment, both the PNPG5 protein and the PNPG2 protein are present in membrane fractions prepared from cells. In another embodiment, the PNPG5

protein is a soluble protein and the PNPG2 protein is present in a membrane of an intact cell. In another embodiment, the PNPG2 protein is a soluble protein and the PNPG5 protein is present in a membrane of an intact cell. In another embodiment, both the PNPG5 protein and the PNPG2 protein are present in membranes of intact cells. In one embodiment, the PNPG5 protein and the PNPG2 protein are present in membranes of different cells. In another embodiment, the PNPG5 protein and the PNPG2 protein are present in the membranes of the same cell. In one embodiment, the cell or cells in which the protein or proteins are present have been subjected to a stress condition corresponding to a painful stimulus.

[0305] In one embodiment, one or both of PNPG5 and PNPG2 proteins are expressed as chimeric/fusion proteins containing an immunodetectable tag (*e.g.*, FLAG™, HA, V5, myc, His₆, Fc, etc.) or a fluorescent/luminescent reporter polypeptide (*e.g.*, GFP, YFP, luciferase, etc.) or one or both proteins are radiolabeled with the same or different radiolabels.

5.11.1. Assays for Detecting and Measuring PNPG5 Binding to Substrates including PNPG2

[0306] The present invention contemplates the use of any of several assays to screen and identify modulators (*e.g.*, inhibitors) of ligand binding to the PNPG5 protein. In one example of an assay, the PNPG5 protein is immobilized on a substrate, and interaction (*i.e.*, binding) with a binding partner is assessed in both the presence and absence of a test compound. In another example, interaction between the PNPG5 protein and its binding partner is assessed in a solution assay, both in the presence and absence of a candidate compound. In either assay, a modulator is identified as a compound that affects (*i.e.*, decreases or increases) binding between the PNPG5 protein and its binding partner. Another useful assay involves a variation of the two-hybrid assay wherein an inhibitor of protein/protein interactions is identified by detection of a signal in a transformed or transfected host cell.

[0307] Candidate compounds to be tested as modulators can be selected from chemical compounds, including libraries of chemical compounds. There are a number of different libraries that can be used for the identification of small molecule modulators, including: (1) chemical libraries, (2) natural product libraries, and (3) 5 combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules. For reviews, see *Science* 1998; 282:63-68 and Myers, *Curr. Opin. Biotechnol.* 1997; 8:701-707.

[0308] PNPG5 modulators contemplated by the invention can be designed, and these include soluble forms of binding partners as well as chimeric or fusion 10 proteins. The term "binding partner" as used herein broadly encompasses non-peptide modulators, peptide modulators (*e.g.*, neuropeptide variants), and antibodies (including monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, bifunctional/bispecific antibodies, humanized antibodies, human antibodies, complementary determining region (CDR)-grafted antibodies, including 15 compounds which include CDR and/or antigen-binding sequences that specifically recognize a polypeptide of the invention, antibody fragments, and modified compounds comprising antibody domains that are immunospecific for the expression product of the PNPG5 gene).

[0309] A number of assays are known in the art that can be used to identify 20 chemical compounds that bind to or otherwise interact with a PNPG5 protein. Such assays are useful, for example, in methods of identifying modulators described herein, or in methods for identifying natural ligands of a PNPG5 protein. Assays that measure binding or other interaction of compounds with PNPG5 include assays that identify compounds that inhibit unfolding or denaturation of a target protein, 25 assays that separate compounds that bind to target proteins through affinity ultrafiltration followed by ion spray mass spectroscopy/HPLC methods or other physical and analytical methods, capillary electrophoresis assays and two-hybrid assays.

[0310] One screening method useful to identify direct binding of test ligands to a target protein relies on distinguishing folded versus unfolded states of a protein, and is described in U.S. Patent No. 5,585,277.

[0311] Another method for identifying ligands of a target protein relies on the screening of combinatorial libraries, as described in Wieboldt *et al.*, *Anal. Chem.* 1997; 69:1683-1691.

[0312] Alternatively, binding interactions can be evaluated indirectly using the yeast two-hybrid system described in Fields *et al.*, *Nature* 1989; 340:245-246 and Fields *et al.*, *Trends in Genetics* 1994; 10:286-292 (see also Vidal and Legrain, *Nucleic Acids Res.* 1999; 27: 919-29), or by using commercially available kits based on the two-hybrid system, such as the MATCHMAKER system (Clontech). PNPG5 may also be used in the PATHCALLING process (CuraGen Corp., New Haven, CT), which employs the yeast two-hybrid system in a high-throughput manner to determine all interactions between the proteins encoded by two large libraries of genes (U.S. Patent No. 6,057,101).

[0313] Where the PNPG5 protein is found to bind to another protein or nucleic acid, the two-hybrid assay can be used to detect agents that interfere with the binding interaction. Expression of the reporter gene can be monitored as different test agents are tested in the system.

[0314] In another specific assay, to identify PNPG5-interacting molecules (*e.g.*, ligands, agonists or antagonists), PNPG5 protein or a biologically active fragment thereof can be labeled with a radiolabel such as ¹²⁵I Bolton-Hunter reagent (Bolton and Hunter, *Biochem. J.* 1973; 133:529-539). Test molecules previously arrayed in the wells of a multi-well plate can be incubated with the labeled PNPG5, washed, and any wells with labeled complex are detected. Data obtained using different concentrations of PNPG5 can be used to calculate values for the number, affinity, and association of PNPG5 with each test molecule.

[0315] Molecules that interact with PNPG5 *in vivo* can be identified by co-immunoprecipitation (*e.g.*, using antibodies against PNPG5 or tag-specific

antibodies or interacting compounds) or affinity chromatography (*e.g.*, using immobilized PNPG5 or its fragment) from cultured cells or DRG tissue lysate. To detect transient interactions, co-immunoprecipitation can be performed after *in vivo* crosslinking (*e.g.*, using formaldehyde). Co-immunoprecipitated or affinity-purified molecules can be further identified using SDS-PAGE and mass spectrometry as described in recent reviews (Wu and Yates, *Nature Biotech.* 2003; 21: 262-267; Graves and Haystead, *Microbiol. Mol. Biol. Rev.* 2002; 66: 39-63)

[0316] The present invention also provides a method of identifying peptide ligands that bind to PNPG5 by phage display method. In phage display, the fusion proteins are incorporated into phage particles such that the particles display the PNPG5-binding peptide on the surface of the phage particle. For example, The Ph.D.-7 Phage Display Peptide Library (Cat#E8100S, New England BioLab, Inc, Beverly, MA) is based on a combinatorial library of random heptapeptides fused to a minor coat protein (pIII) of M13 phage. A library of fusion proteins created and displayed on phage particles can be "panned" against a PNPG5 target (which can be a fragment of PNPG5 protein) to identify test peptides that bind to specific PNPG5 domains. Phages displaying PNPG5-binding peptides are then isolated, and the sequence of the displayed peptide is determined, for example, by sequencing the fusion gene. The sequence of one or more binding peptides can then be compared to the carboxyl-terminal sequences of known proteins to determine which known intracellular proteins have a carboxyl-terminal sequence identical to or similar to the PNPG5-binding peptide(s) to identify cognate protein ligands for the PNPG5.

[0317] To determine critical residues in a PNPG5-binding protein ligand, alanine scanning can be performed. In this method, residues are substituted with a single amino acid, typically an alanine residue, and the effect on PNPG5 binding is assessed. See U.S. Pat. No. 5,580,723; U.S. Pat. No. 5,834,250. Another useful method is construction of a series of truncations (deletions) of PNPG5-binding peptide. A series of truncations may be prepared by truncating the amino terminal amino acids sequentially; in another series, the truncations may begin at the carboxy terminus. As in the case for alanine scanning, the peptides may be synthesized *in vitro* or prepared by recombinant methods. Creating a series of truncations allows

not only the elucidation of those residues critical for binding, but also a determination of the minimal length of peptide to achieve binding. In some cases, truncation will reveal a ligand that binds more tightly than the native ligand; such a peptide may be useful to inhibit PNPG5 ligand interactions *in vivo*.

5 [0318] Based on information obtained from alanine scanning and truncation analysis, the skilled artisan can design and synthesize small molecules, or select small molecule libraries that are enriched in molecules that affect binding between PNPG5 and its ligand. In these assays, PNPG5:binding ligand complexes can be formed in solution, or where one of the binding partners is bound to an insoluble
10 support. The complex can then be separated from a solution, for example, using column chromatography, or can be separated while bound to a solid support by filtration, centrifugation, or using any other well-known techniques. Immobilization of PNPG5 or its ligand on a solid support may facilitate high throughput assays.

[0319] Test compounds can be screened for the ability to affect the
15 interaction of a PNPG5-binding polypeptide with the PNPG5 protein. Screening can be accomplished in any suitable vessel, such as a microtiter plate, test tube, or microcentrifuge tube. Fusion proteins can also be prepared to facilitate testing or separation, where the fusion protein contains an additional domain that allows one or both of the proteins to be bound to a solid support. For example, GST-PNPG5-
20 binding peptide fusion proteins or GST-PNPG5 fusion proteins can be adsorbed onto glutathione sepharose beads or glutathione derivatized microtiter plates, which are then combined with the test compound, or the test compound and either the non-adsorbed PNPG5 protein or PNPG5-binding-peptide, and the mixture incubated under conditions allowing complex formation (*e.g.*, at physiological conditions of
25 salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, and the amount of complex determined either directly or indirectly. Alternatively, the complex can be dissociated from the matrix, and the level of PNPG5 binding or activity determined using standard techniques. Other fusion polypeptide techniques
30 for immobilizing proteins on matrices can also be used in screening assays. Either a PNPG5 binding peptide or its target PNPG5 can be immobilized using biotin-avidin

or biotin-streptavidin systems. Biotinylation can be accomplished using several different reagents, such as biotin-N-hydroxy-succinimide, and immobilized in wells of streptavidin-coated 96 well plates. Alternatively, antibodies reactive with PNPG5-binding peptides or PNPG5, and which do not interfere with binding of a PNPG5-binding peptide to PNPG5 can be immobilized on the solid support. Methods for detecting such complexes, in addition to those described above, include immunodetection using antibodies reactive with PNPG5-binding peptides or target PNPG5.

[0320] To assess the binding affinity of a peptide or other PNPG5 ligand, a competition binding assay can be used, where the ability of the ligand to bind to the PNPG5 protein (and its binding affinity, if desired) is assessed and compared to that of a compound known to bind the PNPG5 protein such as, for example, a consensus peptide sequence determined by phage display or the cognate protein ligand determined as described above.

[0321] Many general methods are known and can be used to identify the binding affinities of PNPG5-binding ligands (*e.g.*, peptides, proteins, small molecules, etc.). For example, binding affinities can be determined as IC₅₀ values using competition ELISAs. For example, in solid phase assays, assay plates may be prepared by coating microwell plates (preferably treated to efficiently absorb protein) with neutravidin, avidin or streptavidin. Non-specific binding sites are then blocked through addition of a solution of bovine serum albumin (BSA) or other proteins (for example, nonfat milk) and then washed, preferably with a buffer containing a detergent, such as Tween-20. A biotinylated PNPG5 ligand (for example, a phage peptide or cognate protein as fusion partner with GST or other such molecule to facilitate purification and detection) is prepared and bound to the plate. Serial dilutions of the ligand to be tested with a PNPG5 protein are prepared and contacted with the bound ligand. The plate coated with the immobilized ligand is washed before adding each binding reaction to the wells and briefly incubated. After further washing, the binding reactions are detected, often using a primary antibody recognizing the non-PNPG5 fusion partner and a secondary antibody with a detectable label (such as horseradish peroxidase (HRP), alkaline phosphatase

(AP), or a fluorescent tag such as fluorescein). The plates are then developed with the appropriate substrate (depending on the label) and the signal quantified, *e.g.*, using a spectrophotometric plate reader. The absorption signal may be fit to a binding curve using a least squares fit. Thus, the ability of various molecules to
5 affect PNPG5 binding to a known PNPG5 ligand can be determined.

[0322] Many variations of the above assay will be apparent to one of skill in the art. For example, instead of avidin-biotin based systems, PNPG5 ligands may be chemically linked to a substrate, or simply adsorbed.

[0323] Aptamers are short oligonucleotide sequences that can be used to
10 recognize and specifically bind to almost any molecule. The systematic evolution of ligands by exponential enrichment (SELEX) process (Ellington and Szostak, *Nature* 1990; 346:818-22; Tuerk and Gold, *Science* 1990; 249:505-10) can be used to find such aptamers. Aptamers have many diagnostic and clinical uses, and may generally be used in any case where an antibody has been used clinically or
15 diagnostically. Once they have been identified, aptamers are less expensive to manufacture than antibodies and can easily be applied in a variety of formats, including in pharmaceutical compositions, bioassays and diagnostic tests (Jayasena, *Clin Chem.* 1999; 45:1628-50). In the competitive ELISA binding assay described above, the screen for candidate aptamers would include incorporating the aptamers
20 into the assay and determining their ability to inhibit PNPG5:ligand binding.

[0324] In a specific embodiment, an antibody may be selected that affects binding of PNPG5 ligand to PNPG5. Examples of such antibodies include polyclonal, monoclonal, single-chain, anti-idiotypic, chimeric Abs, and humanized
25 versions of such antibodies or fragments thereof. Antibodies may be from any species in which an immune response can be raised.

[0325] In conjunction with the screening methods of the present invention, also provided herein are the following features:

(1) a method for determining a ligand or binding partner to the PNPG5 protein of the present invention, which comprises bringing a labeled test compound in contact

with the PNPG5 protein or a fragment thereof and measuring the amount of the labeled test compound bound to the PNPG5 protein or to the fragment thereof;

(2) a method for determining a ligand or binding partner to the PNPG5 protein of the present invention, which comprises bringing a labeled test compound in contact
5 with cells or cell membrane fraction containing the PNPG5 protein, and measuring the amount of the labeled test compound bound to the cells or the membrane fraction; and,

(3) a method for determining a ligand or binding partner to the PNPG5 protein of the present invention, which comprises culturing a transfected cell containing the
10 DNA encoding the PNPG5 protein under conditions that permit or induce expression of the PNPG5 protein, bringing a labeled test compound in contact with the PNPG5 protein expressed on a membrane of said cell, and measuring the amount of the labeled test compound bound to the PNPG5 protein.

[0326] For example, the ligand or binding partner of the PNPG5 protein can
15 be determined by the following procedures. First, a standard PNPG5 protein preparation can be prepared by suspending cells containing the PNPG5 protein, or a membrane fraction from such cells, in a buffer appropriate for use in the determination method. Any buffer that does not inhibit the binding of ligand to the PNPG5 protein can be used. Such buffers include, *e.g.*, a phosphate buffer or a
20 Tris-HCl buffer having a pH of 4 to 10 (preferably a pH of 6 to 8). For the purpose of minimizing non-specific binding, a surfactant such as CHAPS, Tween-80TM (manufactured by Kao-Atlas Inc.), digitonin or deoxycholate and/or various proteins such as bovine serum albumin or gelatin may be added to the buffer. For the purpose of suppressing degradation of the PNPG5 protein or ligand by proteases, a
25 protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Institute, Inc.) and/or pepstatin can be added. A given amount (*e.g.*, 5,000 to 500,000 cpm) of the test compound labeled with [³H], [¹²⁵I], [¹⁴C], [³⁵S] or the like can be added to about 0.01 ml to 10 ml of the solution containing the PNPG5 protein. To determine the amount of non-specific binding (NSB), a reaction mixture
30 containing a large excess of unlabeled test compound is also prepared. The reaction

is carried out at approximately 0 to 50°C, preferably about 4 to 37°C, for about 20 minutes to about 24 hours, and preferably from about 30 minutes to about 3 hours. After completion of the reaction, the cells or membranes containing any bound ligand are separated, *e.g.*, the reaction mixture is filtered through glass fiber filter paper and washed with an appropriate volume of the same buffer. The residual radioactivity on the glass fiber filter paper can be measured by means of a liquid scintillation counter or γ -counter. A test compound exceeding 0 cpm obtained by subtracting NSB from the total binding (B) (B minus NSB) may be selected as a ligand or binding partner of the PNPG5 protein.

10 [0327] According to the present invention, the ligand or binding partner of the PNPG5 protein (including various modulators of PNPG5 activity) can be identified (and its effect further studied) using any method which can be applied to the study of proteins containing LRR domains.

[0328] The art recognizes that agonist-bound proteins can form ternary complexes with other ligands or "accessory" proteins and display altered binding and/or signaling properties in relation to the binary agonist-receptor complex. Accordingly, allosteric sites on the PNPG5 protein represent novel modulator targets and potential drug targets since allosteric modulators possess a number of theoretical advantages over classic orthosteric ligands, such as a ceiling level to the allosteric effect and a potential for greater GPCR subtype-selectivity. Because of the non-competitive nature of allosteric phenomena, the detection and quantification of such effects often rely on a combination of equilibrium binding, nonequilibrium kinetic, and functional signaling assays. For a review see, *e.g.*, Christopoulos and Kenakin, *Pharmacological Reviews*, 2002, 54: 323-74.

25 [0329] Hetero-oligomerization of PNPG5 with other proteins comprising an LRR domain (*e.g.*, PNPG2, AMIGO2, PNPG3/AMIGO3), as well as homo-oligomerization of PNPG5 can be studied, *e.g.*, by using cross-linking and immunoprecipitation with heterologously-expressed differentially epitope-tagged receptors, and/or using biophysical techniques such as bioluminescence and fluorescence resonance energy transfer. (for review see, *e.g.*, Rios *et al.*,

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Pharmacology & Therapeutics, 2001, 92: 71-87). Fluorescence Resonance Energy Transfer (FRET) is described above in relation to TaqMan®. The Bioluminescence Resonance Energy Transfer (BRET) method for the detection of protein-protein interactions (see, *e.g.*, Xu *et al.*, Proc. Natl. Acad. Sci. USA, 96:151-156, 1999; Angers *et al.*, Proc. Natl. Acad. Sci. USA, 97:3684-3689, 2000) measures the transfer of energy between a luminescent donor (*e.g.*, luciferase expressed as a fusion protein with one of the receptors) and a fluorescent acceptor (*e.g.*, YFP expressed as a fusion protein with one of the receptors). Both FRET and BRET can be applied to the detection of PNPG5 hetero- or homo-oligomerization in intact cells, and to study the effect of agonist/antagonist binding on the level of dimerization. Time-resolved FRET employing N-terminally epitope-tagged PNPG5 can be used to detect PNPG5-containing dimers trafficked successfully to the cell surface (for review see, *e.g.*, Milligan *et al.*, Life Sciences, 2003, 74: 181-8; Lee *et al.*, *ibid.*, 173-80). The use of BRET to detect interaction of PNPG5/FLRT3 from *Xenopus laevis* is disclosed in Bottcher *et al.*, Nat Cell Biol. 2004, 6: 38-44.

[0330] PNPG5 dimerization partners and other molecules interacting with PNPG5 in signal transduction pathways can also be identified using yeast two-hybrid screens and proteomics assays. Small molecules that can act as PNPG5 ligands can be further optimized using classical ligand-based design approaches (such as, *e.g.*, virtual screening, pharmacophore modeling, quantitative structure-activity relationship (QSAR), etc.), or by synthesizing and screening combinatorial libraries (for review see, *e.g.*, Klabunde and Hessler, Chembiochem., 2002, 3: 928-44).

[0331] In one embodiment, the amount of the PNPG5 protein associated with the PNPG2 protein is determined by immunoprecipitation or bioluminescence resonance energy transfer (BRET) or mammalian two-hybrid system (Stratagene Cat#211344), or CytoTrap two-hybrid system (Stratagene Cat#217438), or BIACORE methods to detect protein-protein interactions.

5.11.2. Assays for Detecting and Measuring PNPG2 Activity

[0332] In another embodiment, the amount of the PNPG5 protein bound to the PNPG2 protein is determined by measuring a G-protein coupled receptor (GPCR) activity of the PNPG2 protein. According to the present invention, the GPCR activity of PNPG2 can be any GPCR activity known in the art. In a specific embodiment, the GPCR activity of PNPG2 is a change in cAMP levels in the cell. In a specific embodiment, the change in cAMP levels mediated by GPCR activity is measured by the effect on CRE-mediated luciferase activity. In another specific embodiment, the GPCR activity of PNPG2 is a change in calcium levels in the cell.

10 In another specific embodiment, the GPCR activity of PNPG2 is redistribution of an arrestin molecule from the cytoplasm to the plasma membrane.

[0333] In a specific embodiment, such method for determining GPCR activity of PNPG2 is "arrestin-based technology". This technology is based on the fact that, although GPCRs respond to a diverse range of ligands and signal through multiple heterotrimeric G proteins, the inactivation of GPCR signaling is mediated by a limited set of proteins. In particular, the desensitization of the majority of GPCRs is mediated by the binding of two arrestin isoforms, β -arrestin1 and β -arrestin2, that exhibit overlapping substrate specificity. In response to GPCR activation and phosphorylation by GPCR kinases, β -arrestins redistribute from the cytosol to the plasma membrane to bind GPCRs and subsequently target the receptors for internalization via clathrin-coated vesicles. This property of β -arrestins has allowed the development of a green fluorescent protein (GFP)-based assay (Transfluor technology; Norak Biosciences, Inc, Research Triangle Park, NC) to quantitatively monitor the activation or inactivation of GPCRs by confocal microscopy which detects the redistribution of fluorescently labeled arrestins from the cytoplasm to agonist-occupied receptors at the plasma membrane (Oakley *et al.*, *Assay Drug Dev Technol.* 2002; 1(1 Pt 1):21-30; PCT Publication No. WO 2004/038005; Ferguson and Caron, *Methods Mol Biol.* 2004; 237:121-6). Similarly, the InteraX system (Applied Biosystems, Bedford, MA) uses a pair of inactive β -galactosidase (β -gal) deletion mutants as fusion partners to the protein

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inactive β -galactosidase (β -gal) deletion mutants as fusion partners to the protein targets of interest. To monitor GPCR activation, stable cell lines expressing both

GPCR- and β -arrestin- β -gal fusion proteins are generated. Following ligand stimulation, β -arrestin binds to the activated GPCR, and this interaction drives functional complementation of the β -gal mutant fragments. GPCR activation is measured directly by quantitating restored β -gal activity (Yan *et al.*, *J Biomol Screen.* 2002, 7:451-9). As used herein, "arrestin-based technology" may also utilize β -arrestin mutants that significantly improve the assay signal, in the presence or absence of transfected GRKs to enhance the translocation (of arrestin-mediated receptor internalization), and can be detected by BRET (see below) (see Heding, *Expert Rev. Mol. Diagn.* 2004; 4: 403-411).

[0334] In a further alternative, the assay for PNP2 GPCR activity can be based upon the ability of GPCR family proteins to modulate G protein-activated second messenger signal transduction pathways. In one non-limiting embodiment of this invention, GPCR activity of PNP2 can be tested by monitoring the activity of adenylyl cyclase, an enzyme that is known to be part of the downstream signaling pathway of many GPCRs (Voet and Voet, *Biochemistry*, 2nd edition, New York 1995). Adenylyl cyclase catalyzes the conversion of ATP to cAMP (Voet and Voet, *Biochemistry*, 2nd edition, New York 1995). Thus, assays that detect cAMP (*e.g.*, in the presence or absence of a test compound) can be used to monitor PNP2 GPCR activity (see, *e.g.*, Gaudin *et al.*, *J. Biol. Chem.* 1998; 273:4990-4996). For example, a plasmid encoding full-length PNP2 can be transfected into a mammalian cell line (*e.g.*, Chinese hamster ovary (CHO) or human embryonic kidney (HEK-293) cell lines) using methods known in the art. Transfected cells can be grown in multi-well trays in culture medium for 48 hours, the culture medium discarded, and the attached cells gently washed with PBS. The cells can then be incubated in culture medium with or without ligand for 30 minutes, the medium removed, and the cells lysed by treatment with 1 M perchloric acid. The cAMP levels in the lysate can be measured by radioimmunoassay using methods known in the art. Changes in the levels of cAMP in the lysate from cells exposed to ligand compared to those unexposed to ligand are proportional to the activity of the ligand being tested present in the transfected cells. Alternatively, the change in cAMP levels mediated by GPCR activity can be measured by the effect on CRE-mediated

expression of a reporter gene such as β -galactosidase, GFP or luciferase (*e.g.*, as disclosed in **Figures 9A-B** and Examples Section, below).

[0335] In yet another non-limiting embodiment of this invention, the GPCR activity of PNPG2 can be tested by monitoring the activity of phospholipase C, another enzyme that responds to signals from some GPCRs. Phospholipase C hydrolyzes the phospholipid, PIP₂, releasing two intracellular messengers: diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP₃) (Voet and Voet, *Biochemistry*, 2nd edition, New York 1995). Accordingly, assays that detect DAG and/or IP₃ accumulation (*e.g.*, in the presence or absence of a test compound) can be used to monitor the GPCR activity of PNPG2.

[0336] For example, to measure changes in inositol phosphate levels, the cells are grown in 24-well plates containing 1x10⁵ cells/well and incubated with inositol-free media and [³H]myoinositol, 2 mCi/well, for 48 hr. The culture medium is removed, and the cells washed with buffer containing 10 mM LiCl followed by addition of ligand. The reaction is stopped by adding perchloric acid. Inositol phosphates are extracted and separated on Dowex AG1-X8 (Bio-Rad) anion exchange resin, and the total labeled inositol phosphates counted by liquid scintillation. Changes in the levels of labeled inositol phosphate from cells exposed to ligand compared to those unexposed to ligand are proportional to the activity of PNPG2 present in the transfected cells.

[0337] The GPCR activity of PNPG2 may also be tested by measuring calcium mobilization, MAP kinase activity, or GTP γ S binding.

[0338] The test compound can be a small organic or inorganic molecule, a polypeptide (*e.g.*, a ligand or ligand derivative, or an antibody, or antibody fragment, or other immunospecific molecule), an oligonucleotide molecule, a polynucleotide molecule, or a chimera or derivative thereof. The sources for potential agents to be screened include natural sources, such as cell extracts (*e.g.*, from animal, bacterial, fungal, algal, insect, or plant sources) and synthetic sources, such as chemical compound libraries.

[0339] Assays contemplated by these screening methods can be performed in conventional or HTS formats.

[0340] High-throughput screening (HTS) assays useful in the screening methods of the present invention include cell-based and cell-free assays directed
5 against individual protein targets. Several types of automated assays that have been developed in recent years enable the screening of tens of thousands of compounds in a short period of time (see, *e.g.*, U.S. Patent Nos. 5,585,277, 5,679,582, and 6,020,141). Such HTS methods are particularly preferred.

[0341] One of the preferred high throughput approaches for the analysis of
10 PNPG5 activity is flow cytometry, which offers real-time collection of multicomponent data, and a high throughput sampling rate. For example, a screening platform comprising a dextran bead allowing different surface chemistries on the beads can be used (Waller *et al.*, Combinatorial Chemistry & High Throughput Screening, 2003, 6: 389-97). Dextran beads can be either ligand-
15 labeled or have epitope-linked PNPG5 attached to the bead surface, enabling several molecular assemblies to be constructed and analyzed.

5.11.3. Studying the Biological Activities of PNPG5

[0342] The present invention further provides various methods for studying biological activities of the PNPG5 protein. The biological activity of the PNPG5
20 protein can be studied using intact cells that express an endogenously encoded PNPG5 protein, or intact cells that have been genetically engineered to express or overexpress a PNPG5 protein. The cells may express the PNPG5 protein constitutively or in response to a specific stimulus or treatment). Alternatively, the PNPG5 protein can be present in membrane fractions prepared from any of such
25 cells, or the PNPG5 protein can be isolated and unassociated with a cellular membrane. Various functional features of the PNPG5 protein that can be used to determine its biological activity include, without limitation, activation of PNPG5-interacting G protein(s), formation of dimers and oligomers, and interaction with auxiliary membranes and cytosolic proteins.

[0343] A biological activity of the PNPG5 protein can be studied by measuring in a cell that expresses the PNPG5 protein the activities that promote or suppress the production of an "index substance" (*e.g.*, arachidonic acid release, acetylcholine release, intracellular Ca^{2+} release, intracellular cAMP production, intracellular cGMP production, or inositol phosphate production), change in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, pH reduction, etc.

[0344] PNPG5-mediated activities can be determined by any known method. For example, cells containing the PNPG5 protein can first be cultured on a multi-well plate, etc. Prior to activity determination, the medium can be replaced with fresh medium or with an appropriate non-cytotoxic buffer, followed by incubation for a given period of time in the presence of a test compound, etc. Subsequently, the cells can be extracted or the supernatant can be recovered and the resulting product can be quantified by appropriate procedures. Where it is difficult to detect the production of the "index substance" for the cell-stimulating activity due to a degrading enzyme contained in the cells, an inhibitor against such a degrading enzyme may be added prior to the assay. For detecting activities such as the cAMP production suppression activity, the baseline production in the cells can be increased by forskolin or the like and the suppressing effect on the increased baseline production may then be detected.

[0345] According to Kuja-Panula *et al.* (*J. Cell. Biol.* 2003, 160:963-973), immobilized AMIGO-Ig fusions can promote neurite extensions of hippocampal neurons. Additionally, soluble AMIGO-Ig inhibits fasciculation in hippocampal neurons. Biological activities related to these known functions can be further studied as part of this invention.

[0346] The above-identified screening methods can be used to identify a candidate compound that can be used to treat a condition that can be treated by modulating the expression and/or activity of a PNPG5 protein of the present invention.

30 5.12. Methods of Treatment

[0347] The present invention further provides a method for treating a condition by modulating expression of a PNPG5-encoding nucleic acid molecule, or by modulating expression of a PNPG5 protein, or by modulating an activity of a PNPG5 protein comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound that modulates expression of a PNPG5-encoding nucleic acid molecule, or that modulates expression of a PNPG5 protein, or that modulates activity of a PNPG5 protein, respectively.

[0348] In another embodiment, the present invention provides a method for treating a condition comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound that modulates binding between a PNPG2 protein and a PNPG5 protein or another LRR-domain containing protein.

[0349] For example, administration of a soluble PNPG5 may have a therapeutic effect by interacting with cellular membrane-associated PNPG2 and reducing cAMP production (similar action to opioid agonists at opioid receptors).

[0350] Conditions that can be treated using the method disclosed herein include a pain condition or a pain-related disorder selected without limitation from chronic pain, nociceptive pain, neuropathic pain (including all types of hyperalgesia (*i.e.*, sensation of more pain than the stimulus would warrant) and allodynia (*i.e.*, a condition in which ordinarily painless stimuli induce the experience of pain)), inflammatory and cancer pain, addiction, seizure (including epilepsy), stroke or ischemia, neurodegenerative disorder (*e.g.*, Parkinson's disease, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), or Huntington's chorea), headache (*e.g.*, general, migraine, cluster or tension), anxiety, depression, asthma, rheumatic disease, osteoarthritis, retinopathy, inflammatory eye disorders, pruritis, ulcer (gastric or duodenal), gastric lesions (*e.g.*, induced by a necrotizing agent), uncontrollable urination (*e.g.*, incontinence), an inflammatory or unstable bladder disorders, inflammatory bowel disease (*e.g.*, Crohn's disease and ulcerative colitis), irritable bowel syndrome (IBS) including irritable bowel disease (IBD), gastroesophageal reflux disease (GERD), functional dyspepsia (*e.g.*, ulcer-like dyspepsia, dysmotility-like dyspepsia, functional heartburn, and non-ulcer

dyspepsia), functional chest pain of presumed oesophageal origin, functional dysphagia, non-cardiac chest pain, symptomatic gastroesophageal disease, gastritis, aerophagia, functional constipation, functional diarrhea, burbulence, chronic functional abdominal pain, recurrent abdominal pain (RAP), functional abdominal bloating, functional biliary pain, functional incontinence, functional ano-rectal pain, chronic pelvic pain, pelvic floor dyssnergia, unspecified functional ano-rectal disorder, cholecystalgia, interstitial cystitis, dysmenorrhea, and dyspareunia.

[0351] In another preferred embodiment, the condition treated by the method of the present invention is chronic pain. In a preferred embodiment, the condition treated by the method of the present invention is neuropathic pain.

[0352] The term "therapeutically effective amount" is used here to refer to: (i) an amount or dose of a compound sufficient to detectably change the level of expression of a PNPG5-encoding nucleic acid or protein in a subject; or (ii) an amount or dose of a compound sufficient to detectably change the level of activity of a PNPG5 protein in a subject; or (iii) an amount or dose of a compound sufficient to cause a detectable improvement in a clinically significant symptom or condition (*e.g.*, amelioration of pain) in a subject.

[0353] A candidate compound useful in conducting a therapeutic method of the present invention is advantageously formulated in a pharmaceutical composition with a pharmaceutically acceptable carrier. The candidate compound may be designated as an active ingredient or therapeutic agent for the treatment of pain or other indication.

[0354] The concentration of the active ingredient depends on the desired dosage and administration regimen, as discussed below. Suitable dose ranges of the active ingredient are from about 0.01 mg/kg to about 1500 mg/kg of body weight per day.

[0355] Therapeutically effective compounds can be provided to the patient in standard formulations, and may include any pharmaceutically acceptable additives, such as excipients, lubricants, diluents, flavorants, colorants, buffers, and

disintegrants. The formulation may be produced in useful dosage units for administration by oral, parenteral, transmucosal, intranasal, rectal, vaginal, or transdermal routes. Parental routes include intravenous, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, intrathecal, and intracranial administration.

[0356] The pharmaceutical composition may also include other biologically active substances in combination with the candidate compound. Such substances include but are not limited to opioids, non-steroidal anti-inflammatory drugs (NSAIDs), and other analgesics.

[0357] The pharmaceutical composition can be added to a retained physiological fluid such as blood or synovial fluid. For CNS administration, a variety of techniques are available for promoting transfer of the therapeutic agent across the blood brain barrier, including disruption by surgery or injection, co-administration of a drug that transiently opens adhesion contacts between CNS vasculature endothelial cells, and co-administration of a substance that facilitates translocation through such cells.

[0358] In another embodiment, the active ingredient can be delivered in a vesicle, particularly a liposome.

[0359] In another embodiment, the therapeutic agent can be delivered in a controlled release manner. For example, a therapeutic agent can be administered using intravenous infusion with a continuous pump, in a polymer matrix such as poly-lactic/glutamic acid (PLGA), in a pellet containing a mixture of cholesterol and the active ingredient (SilasticR™; Dow Corning, Midland, MI; see U.S. Patent No. 5,554,601), by subcutaneous implantation, or by transdermal patch.

25 **5.13. Neuronal Cell Cultures**

[0360] DRG neuronal cell cultures are useful in practicing various aspects of the present invention, and can be prepared using ordinary techniques known in the art. The screening methods and biological activity assays of the present invention

can use cultured cells or cell lines to screen for candidate compounds useful as therapeutic agents. The cells are preferably neurons or other cells present in CNS or PNS.

[0361] Cultured post-mitotic or neuronal precursors can be obtained using various methods. As one example, primary neurons or neural progenitor cells are isolated and cultured according to methods known in the art (see, *e.g.*, U.S. Patent No. 5,654,189). Examples of neurons useful for carrying out the methods of the present invention include brain or spinal cord neurons collected from mammals, and neuronal cell lines grown in the presence of growth factors such as NGF (nerve growth factor), IGF (insulin-like growth factor), etc.

[0362] For example, DRG neurons from rats can be dissociated following the procedure of Caldero *et al.*, *J. Neurosci.* 1998; 18: 356-370. Following dissociation, neurons can be placed in tissue culture dishes or micro-wells coated, *e.g.*, with ornithine-laminin, in medium supplemented with glutamine, fetal bovine serum (FBS), putrescine, sodium selenite, progesterone and antibiotics (see, *e.g.*, Baudet *et al.*, *Development* 2000; 127: 4335-4344). Growth factors such as NGF, FGF (fibroblast growth factor), EGF (epidermal growth factor), interleukin 6 (*Ann. Rev. Pharmacol. Toxicol.* 1991; 31: 205-228), IGF (*J. Cell Biol.* 1986; 102: 1949-1954), and those described in *Cell Culture in the Neurosciences*, New York: Plenum Press, 1955, pp. 95-123, can also be included.

[0363] In another embodiment, transformed neuronal cell lines, such as those created with tetracarcinoma cell lines, can also be used.

[0364] In another embodiment, clonal cell lines can be isolated from a conditionally immortalized neural precursor cell line (see, *e.g.*, U.S. Patent No. 6,255,122). A skilled artisan will readily appreciate that cells or cell cultures used in the methods of this invention should be carefully controlled for parameters such as cell passage number, cell density, the methods by which the cells are dispensed, and growth time after dispensing.

5.14. Animal Models of Pain

[0365] As specified above, the diagnostic and screening methods of the present invention can be conducted in (i) any cell derived from a tissue of an organism experiencing a feeling of pain or a pain-related condition or (ii) any cell grown *in vitro* in tissue culture under specific conditions that mimic some aspect of tissue conditions in an organism experiencing a feeling of pain (*e.g.*, nerve injury, inflammation, viral infection, etc.). Particularly useful for the diagnostic and screening methods of the present invention are cells (especially neural cells) derived from animal models of pain and related disorders. The PNPG5 gene of the invention was identified using a rat spinal nerve ligation (SNL) model of neuropathic pain (Kim and Chung, *Pain* 1992; 50: 355-363), which is a particularly useful source of cells in the methods of the present invention. Some of the additional useful models are described below.

5.14.1. FCA Injection Model

[0366] A chronic pain condition can be reproduced in mice or rats by the injection of Freund's complete adjuvant (FCA) containing heat-killed *Mycobacterium* into the base of the tail or into the hind footpads (Colpaert *et al.*, *Life Sci.* 1980; 27: 921-928; De Castro Costa *et al.*, *Pain* 1981; 10: 173-185; Larson *et al.*, *Pharmacol. Biochem Behav.* 1986; 24: 9-53).

[0367] For example, a chronic pain condition can be induced by intradermal injection of 50 µl of 50% FCA into one hindpaw, wherein undiluted FCA consists of 1 mg/ml heat-killed and dried *Mycobacterium*, each ml of vehicle contains 0.85 ml paraffin oil + 0.15 ml mannide monooleate (Sigma, St. Louis, MO), and FCA is diluted in a ratio of 1:1 (vol:vol) with 0.9% saline. Intradermal injection can be performed under isoflurane/O₂ inhalation anesthesia. The treated and control (*e.g.*, given an intradermal injection of 0.9% saline, or the same adjuvant lacking heat-killed *Mycobacterium*) animals can be tested between 24 and 72 hours following FCA injection.

[0368] FCA injection causes an inflammation (in the case of injection into the base of the tail, wide-spread joint inflammation mimicking rheumatoid arthritis)

that lasts for several days, and is evidenced by the classical signs of inflammation (erythema, edema, heat), as well as hyperalgesia (*e.g.*, to thermal and mechanical stimuli) and allodynia (Fundytus *et al.*, *Pharmacol Biochem & Behav* 2002; 73: 401-410; Binder *et al.*, *Anesthesiology* 2001; 94: 1034-1044). The pain sensitivity (*i.e.*, alterations in nociceptive thresholds) can then be measured in the injected and neighboring regions by decreases in response latency (compared to control animals injected with the same adjuvant lacking heat-killed *Mycobacterium*, or 0.9% saline). For example, thermal hyperalgesia can be assessed by applying focused radiant heat to the plantar surface of the hindpaw and measuring the latency for the animal to withdraw its paw from the stimulus (Hargreaves *et al.*, *Pain* 1988; 32: 77-88; D'Amour and Smith, *J. Pharmacol. Exp. Ther.* 1941; 72: 74-79; *see also* the hot-plate assay described by Eddy and Leimbach, *J. Pharmacol. Exp. Ther.* 1953; 107: 385-393). A decrease in the paw withdrawal latency following FCA injection indicates thermal hyperalgesia. Mechanical hyperalgesia can be assessed with the paw pressure test, where the paw is placed on a small platform, and weight is applied in a graded manner until the paw is completely withdrawn (Stein, *Biochemistry & Behavior* 1988; 31: 451-455, *see also* the Examples section, below). Mechanical allodynia can be also assessed by applying thin filaments (von Frey hairs) to the plantar surface of the hindpaw and determining the response threshold for paw withdrawal (*see* Dixon, *J. Am Stat. Assoc.* 1965; 60: 967-978).

5.14.2. Sciatic Nerve Injury Models

[0369] The first animal model of neuropathic pain to be developed was the simple cutting of the sciatic nerve, termed "axotomy" (Wall *et al.*, *Pain* 1979; 7: 103-111). Following axotomy, neuromas form at the ends of the cut nerve. With this type of injury, self-mutilation of the injured foot, termed "autotomy", is often observed.

[0370] In this model, a unilateral nerve injury is induced by exposing and cutting one sciatic nerve. The ends of the cut sciatic nerve are then ligated to prevent re-growth. Surgery is performed under isoflurane/O₂ anesthesia. The wound is closed with 4-0 Vicryl, dusted with antibiotic powder, and the animals are

allowed to recover on a warm heating pad before being returned to their home cages. Sham-operated animals are used as a control. Sham-operation consists of exposing but not injuring one sciatic nerve. Animals are observed for up to two weeks to assess pain behaviors. Animals are tested with the thermal and mechanical tests as described above.

[0371] One of the most commonly used experimental animal models for neuropathic pain is the chronic constriction injury (CCI) where four loose ligatures are tied around the sciatic nerve (Bennett and Xie, *Pain* 1988; 33: 87-107). One disadvantage of this model is the introduction of foreign material into the wound, which causes a local inflammatory reaction, whereas hyperalgesia does not have to be associated with inflammation. Thus, the distinction between the neuropathic and the inflammatory component of pain is difficult in this model. In order to produce a pure nerve injury model without an epineurial inflammatory component due to foreign material, Lindenlaub and Sommer (*Pain* 2000; 89: 97-106) have recently performed a partial sciatic nerve transection (PST) in rats. These rats developed thermal hyperalgesia and mechanical allodynia comparable to the CCI model. In both models, animals' thermal withdrawal thresholds are commonly assessed by response to radiant heat on the plantar surface of the hindpaw (Hargreaves *et al.*, *Pain* 1988; 32: 77-88). Mechanical hypersensitivity is commonly determined by measuring the withdrawal thresholds to von Frey hairs (Dixon, *J. Am Stat. Assoc.* 1965; 60: 967-978).

[0372] Decosterd and Woolf have recently developed a variant of partial denervation, the spared nerve injury model (Decosterd and Woolf, *Pain* 2000, 87: 149-58). This model involves a lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact. The spared nerve injury model differs from the SNL, CCI and PST models in that the co-mingling of distal intact axons with degenerating axons is restricted, and it permits behavioral testing of the non-injured skin territories adjacent to the denervated areas. The spared nerve injury model results in early (less than 24 hours), prolonged (greater than 6 months), robust (all animals are responders) behavioral modifications. The mechanical sensitivity (as determined, *e.g.*, by

sensitivity to von Frey hairs and pinprick test) and thermal (hot and cold) responsiveness is increased in the ipsilateral sural and to a lesser extent saphenous territories, without any change in heat thermal thresholds.

[0373] Partial sciatic nerve ligation is yet another sciatic nerve injury model
5 (Seltzer *et al.*, Pain 1990, 43: 205-218). In mammals, *e.g.* rats, about half of the sciatic nerves high in the thigh are unilaterally ligated in this model. According to Seltzer *et al.*, rats of this model develop a guarding behavior of the ipsilateral hindpaw and lick it often. These behaviors are observed within a few hours after the operation and for several months thereafter. Allodynia, thermal hyperalgesia,
10 and mechanical hyperalgesia are each observed in this model according to Seltzer *et al.* The partial sciatic nerve ligation model may be used when addressing hypotheses concerning causalgiform pain disorders.

5.14.3. Cancer Pain Models

[0374] The models of neuropathic pain described above involve acute or sub-
15 acute insult of the peripheral nerve, and do not necessarily reflect gradual but progressive insult of the nerve, which is expected to occur in such common neuropathic pain conditions as neuropathic cancer pain. Neuropathic cancer pain can, however, be, reproduced by inoculating Meth A sarcoma cells to the immediate proximity of the sciatic nerve in BALB/c mice (Shimoyama *et al.*, Pain 2002; 99:
20 167-174). The tumor grows predictably with time and gradually compresses the nerve, thereby causing thermal hyperalgesia (as determined, *e.g.*, by paw withdrawal latencies to radiant heat stimulation), mechanical allodynia (as determined, *e.g.*, by sensitivity of paws to von Frey hairs), and signs of spontaneous pain (as detected, *e.g.*, by spontaneous lifting of the paw).

25 [0375] A rat model of bone cancer pain was also recently reported (Medhurst *et al.*, Pain 2002; 96: 129-40). In this model, Sprague-Dawley rats receive intra-tibial injections of 3×10^3 or 3×10^4 syngeneic MRMT-1 rat mammary gland carcinoma cells, which produce rapidly expanding tumors within the boundaries of the tibia, causing severe remodeling of the bone. Rats receiving intra-

tibial injections of MRMT-1 cells develop behavioral signs indicative of pain, including the gradual development of mechanical allodynia and mechanical hyperalgesia/reduced weight bearing on the affected limb, beginning on day 12-14 or 10-12 following injection of 3×10^3 or 3×10^4 cells, respectively. These symptoms are not observed in rats receiving heat-killed cells or vehicle. Acute treatment with morphine produces a dose-dependent reduction in the response frequency of hind paw withdrawal to von Frey hairs as well as reduction in the difference in hind limb weight bearing.

5.14.4. Incisional Model of Post-Operative Pain

[0376] Brennan and colleagues have developed an animal model of post-operative pain (Brennan *et al.*, *Pain* 1996; 64: 493-501), which involves making a surgical incision on the plantar aspect of the rat hindpaw. The mechanical hyperalgesia that is observed in this rat model parallels the time course of pain in post-operative patients, and is alleviated by systemic and intrathecal (i.t.) morphine (Zahn *et al.*, *Anesthesiology* 1997; 86: 1066-1077).

[0377] Specifically, a 1 cm incision is made in the plantar surface of one hindpaw under isoflurane/O₂ inhalation anesthesia. The incision is closed with two sutures using 4-0 Vicryl. Rats are allowed to recover in their home cages. Naïve rats are used as control animals. Mechanical and thermal sensitivity is measured 24 hours after injury, *e.g.*, as described above.

5.15. Use of Arrays

[0378] In one embodiment, the differentially expressed gene of the present invention, *i.e.*, the PNPG5 gene, will be used in screening methods comprising microarrays.

5.15.1. Cell-Based Arrays

[0379] Cell-based arrays combine the technique of cell culture in conjunction with the use of fluidic devices for measurement of cell response to test compounds in a sample of interest, screening of samples for identifying molecules that induce a

desired effect in cultured cells, and selection and identification of cell populations with novel and desired characteristics. High-throughput screens (HTS) can be performed on fixed cells using fluorescent-labeled antibodies, biological ligands and/or nucleic acid hybridization probes, or on live cells using multicolor
5 fluorescent indicators and biosensors. The choice of fixed or live cell screens depends on the specific cell-based assay required.

[0380] There are numerous single- and multi-cell-based array techniques known in the art. Recently developed techniques such as micro-patterned arrays (described, *e.g.*, in International PCT Publications WO 97/45730 and WO
10 98/38490) and microfluidic arrays provide valuable tools for comparative cell-based analysis. Transfected cell microarrays are a complementary technique in which array features comprise clusters of cells overexpressing defined cDNAs. Complementary DNAs cloned in expression vectors are printed on microscope slides, which become living arrays after the addition of a lipid transfection reagent
15 and adherent mammalian cells (Bailey *et al.*, *Drug Discov. Today* 2002; 7(18 Suppl): S113-8). Cell-based arrays are described in detail in, *e.g.*, Beske, *Drug Discov. Today* 2002; 7(18 Suppl): S131-5; Sundberg *et al.*, *Curr. Opin. Biotechnol.* 2000; 11: 47-53; Johnston *et al.*, *Drug Discov. Today* 2002; 7: 353-63; U.S. Patents No. 6,406,840 and 6,103,479, and U.S. published patent application No.
20 2002/0197656. For cell-based assays specifically used to screen for modulators of ligand-gated ion channels, see Mattheakis *et al.*, *Curr. Opin. Drug Discov. Devel.* 2001; 1: 124-34; and Baxter *et al.*, *J. Biomol. Screen.* 2002; 7: 79-85.

5.15.2. Protein Arrays

[0381] Protein arrays are solid-phase, ligand binding assay systems using
25 immobilized proteins on surfaces that are selected from glass, membranes, microtiter wells, mass spectrometer plates, and beads or other particles. The ligand binding assays using these arrays are highly parallel and often miniaturized. Their advantages are that they are rapid, can be automated, are capable of high sensitivity, are economical in their use of reagents, and provide an abundance of data from a
30 single experiment.

[0382] Automated multi-well formats are the best-developed HTS systems. Automated 96-well plate-based screening systems are the most widely used. The current trend in plate based screening systems is to reduce the volume of the reaction wells further, thereby increasing the density of the wells per plate (96 wells to 384 wells, and 1,536 wells per plate). The reduction in reaction volumes results in increased throughput, dramatically decreased bioreagent costs, and a decrease in the number of plates that need to be managed by automation. For a description of protein arrays that can be used for HTS, see, *e.g.*, U.S. Patents No. 6,475,809; 6,406,921; and 6,197,599; and International Publications No. WO 00/04389 and WO 00/07024.

[0383] For construction of arrays, sources of proteins include cell-based expression systems for recombinant proteins, purification from natural sources, production *in vitro* by cell-free translation systems, and synthetic methods for peptides. For capture arrays and protein function analysis, it is important that proteins are correctly folded and functional. This is not always the case, *e.g.*, where recombinant proteins are extracted from bacteria under denaturing conditions, whereas other methods (isolation of natural proteins, cell free synthesis) generally retain functionality. However, arrays of denatured proteins can still be useful in screening antibodies for cross-reactivity, identifying auto-antibodies, and selecting ligand binding proteins.

[0384] The immobilization method used should be reproducible, applicable to proteins of different properties (size, hydrophilic, hydrophobic), amenable to high throughput and automation, and compatible with retention of fully functional protein activity. Both covalent and non-covalent methods of protein immobilization can be used. Substrates for covalent attachment include, *e.g.*, glass slides coated with amino- or aldehyde-containing silane reagents (Telechem). In the Versalinx™ system (Prolinx), reversible covalent coupling is achieved by interaction between the protein derivatized with phenyldiboronic acid, and salicylhydroxamic acid immobilized on the support surface. Covalent coupling methods providing a stable linkage can be applied to a range of proteins. Non-covalent binding of unmodified

protein occurs within porous structures such as HydroGel™ (PerkinElmer), based on a 3-dimensional polyacrylamide gel.

5.15.3. Detection

[0385] For detection of molecules using screening assays, a molecule (*e.g.*,
5 an antibody or polynucleotide probe) can be detectably labeled with an atom (*e.g.*,
radionuclide), detectable molecule (*e.g.*, fluorescein), or complex that, due to its
physical or chemical property, serves to indicate the presence of the molecule. A
molecule can also be detectably labeled when it is covalently bound to a “reporter”
molecule (*e.g.*, a biomolecule such as an enzyme) that acts on a substrate to produce
10 a detectable product. Detectable labels suitable for use in the present invention
include any composition detectable by spectroscopic, photochemical, biochemical,
immunochemical, electrical, optical or chemical means. Labels useful in the present
invention include, but are not limited to, biotin for staining with labeled avidin or
streptavidin conjugate, magnetic beads (*e.g.*, Dynabeads™), fluorescent dyes (*e.g.*,
15 fluorescein, fluorescein-isothiocyanate (FITC), Texas red, rhodamine, green
fluorescent protein, enhanced green fluorescent protein, lissamine, phycoerythrin,
Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, FluorX [Amersham], SyBR Green I & II
[Molecular Probes], and the like), radiolabels (*e.g.*, ³H, ¹²⁵I, ³⁵S, ¹⁴C, or ³²P),
enzymes (*e.g.*, hydrolases, particularly phosphatases such as alkaline phosphatase,
20 esterases and glycosidases, or oxidoreductases, particularly peroxidases such as
horse radish peroxidase, and the like), substrates, cofactors, inhibitors,
chemiluminescent groups, chromogenic agents, and colorimetric labels such as
colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex,
etc.) beads. Examples of patents describing the use of such labels include U.S.
25 Patents No. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149;
and 4,366,241.

[0386] Means of detecting such labels are known to those of skill in the art.
For example, radiolabels and chemiluminescent labels can be detected using
photographic film or scintillation counters; fluorescent markers can be detected
30 using a photo-detector to detect emitted light (*e.g.*, as in fluorescence-activated cell

sorting); and enzymatic labels can be detected by providing the enzyme with a substrate and detecting, *e.g.*, a colored reaction product produced by the action of the enzyme on the substrate.

[0387] The present invention is further described by way of the following particular examples. However, the use of such examples is illustrative only and is not intended to limit the scope or meaning of this invention or of any exemplified term. Nor is the invention limited to any particular preferred embodiment(s) described herein. Indeed, many modifications and variations of the invention will be apparent to those skilled in the art upon reading this specification, and such “equivalents” can be made without departing from the invention in spirit or scope. The invention is therefore limited only by the terms of the appended claims, along with the full scope of equivalents to which the claims are entitled.

6. EXAMPLE: PNPG5 Gene Expression is Modulated in a Neuropathic Pain Model

15 [0388] The present inventors have identified a novel gene PNPG5. The change in PNPG5 gene expression in the SNL model of neuropathic pain parallels change in expression of several genes known to be molecular mediators of pain, thereby linking PNPG5 to a role in mediating or responding to pain.

6.1. Preparation of Neuropathic Pain Model

20 [0389] Rats having the L5-L6 spinal nerves ligated (SNL) according to the method of Kim and Chung, *Pain* 1992; 50:355-63 were used in this experiment. Briefly, nerve injury was induced by tight ligation of the left L5 and L6 spinal nerves, producing symptoms of neuropathic pain as described below. The advantage of this model is that it allows the investigation of dorsal root ganglia that are injured (L5 and L6) versus dorsal root ganglia that are not injured (L4). Thus, it is possible to see changes in gene expression specifically in response to nerve injury. Surgery was performed under isoflurane/O₂ inhalation anesthesia. Following induction of anesthesia, a 3 cm incision was made just lateral to the spinal vertebrae. The left paraspinal muscles were separated from the spinous
25
30 process at the L4-S2 levels. The L6 transverse process was carefully removed with

a pair of small rongeurs to visually identify the L4-L6 spinal nerves. The left L5 and L6 spinal nerves were isolated and tightly ligated with 7-0 silk suture. A complete hemostasis was confirmed, and the wound was sutured using non-absorbable sutures, such as 4-0 Vicryl.

5 [0390] Both naïve and sham-operated animals were used as controls. Sham-operation consisted of exposing the spinal nerves without ligation or manipulation. After surgery, animals were weighed and administered a subcutaneous (s.c.) injection of Ringers lactate solution. Following injection, the wound area was dusted with antibiotic powder and the animals were kept on a warm pad until
10 recovery from anesthesia. Animals were then returned to their home cages until behavioral testing. The naïve control group consisted of rats that were not operated on (naïve). Eight to twelve rats in each group were evaluated.

[0391] Some rats from the SNL and naïve groups were also treated with gabapentin (GPN) as described below. Gabapentin (GPN), an anti-convulsant, has
15 been shown in the clinic to be effective for treating neuropathic pain (Melleger *et al.*, *Clin. J. Pain* 2001; 17: 284-295; Rose and Kam, *Anaesthesia* 2002; 57: 451-462).

[0392] The present inventors set out to use the L4, L5 and L6 DRGs from SNL model of neuropathic pain to identify genes involved in mediating and
20 responding to pain (including genes affected by GPN treatment) by using expression profiling, which is based on identifying probes on a "genome-scale" microarray that are differentially expressed in SNL DRGs as compared to DRGs of naïve and sham-operated animals.

Table 1 summarizes five experimental groups consisting of sham surgery, naïve or SNL surgery with or without GPN treatment:

Group Number	Experimental Group Name	Surgery	Drug Treatment
1	naïve + vehicle	none performed	vehicle
2	naïve + GPN	none performed	gabapentin
3	sham + vehicle	sham	vehicle
4	SNL + vehicle	SNL	vehicle
5	SNL + GPN	SNL	gabapentin

6.2. Behavioral Testing

5 [0393] Mechanical sensitivity was assessed using the paw pressure test. This test measures mechanical hyperalgesia. Hind paw withdrawal thresholds (“PWT”) (measured in grams) in response to a noxious mechanical stimulus were determined using an analgesymeter (Model 7200, commercially available from Ugo Basile of Italy), as described in Stein, *Biochemistry & Behavior* 1988; 31: 451-455. The rat’s

10 paw was placed on a small platform, and weight was applied in a graded manner up to a maximum of 250 grams. The endpoint was taken as the weight at which the paw was completely withdrawn. PWT was determined once for each rat at each time point, and only the injured ipsilateral paw (*i.e.*, the hind paw on the same side of the animal as the ligation in SNL animals, or the side of the animal where the

15 nerve was exposed but not injured in sham-operated animals) was used in the test. For naïve animals, the left paw or the side that “would have been” subjected to surgery (herein also referred to as “ipsilateral”) was used for the test.

[0394] Rats were tested prior to injury (SNL or sham surgery; naïve rats were tested at the same time) to determine a baseline, or normal, PWT. To verify

20 that the surgical procedure was successful, rats were again tested at 12-14 days after surgery. At this time, rats with an SNL injury should exhibit a significantly reduced PWT compared to their baseline PWT, while sham-operated and naïve rats should have PWT that is not significantly different from their baseline PWT. Only rats that

met these criteria were included in further behavioral testing and the gene expression study.

[0395] Rats that met the behavior criteria were divided into the treatment groups (described above): 1) naïve + vehicle; 2) naïve + GPN; 3) sham + vehicle; 5 4) SNL + vehicle; 5) SNL + GPN (Table 1). Vehicle (0.9% saline) and GPN (dissolved in 0.9% saline) were administered intraperitoneally (i.p.) in a volume of 2 ml/kg. The dose of GPN was 100 mg/kg. The rats in the above treatment groups were treated each day for 7 days (with either vehicle or GPN as per their group), and on the last (7th) treatment day (corresponding to 19-21 days post surgery), rats 10 were again assessed for mechanical sensitivity using the paw pressure test described above, in particular to confirm the reversal of neuropathic pain with GPN treatment. Following testing, tissues were collected as described below. See **Figure 6** for a summary of the experimental timelines for surgery, treatment, and testing.

6.3. Determining Gene Expression Profiles in the SNL Model

6.3.1. Tissue Collection and RNA Preparation

[0396] Eight to twelve rats meeting behavioral criteria for the five experimental groups described above were sacrificed, and the following tissues were collected separately: ipsilateral and contralateral dorsal root ganglia (DRG) for L4, L5 and L6. Samples were rapidly frozen on dry ice. Next, for each experimental 20 group and tissue (5 groups x 6 tissues = 30 total), the samples were separated into two pools (Pool 1 and Pool 2), consisting of half or 4-6 animals each.

[0397] Total RNA from each tissue sample pool was prepared using Tri-Reagent (Sigma, St. Louis, MO). Total RNA was quantified by measuring absorption at 260 nm. RNA quality was assessed by measuring absorption at 260 25 nm/280 nm and by capillary electrophoresis on an RNA Lab-on-chip using Bioanalyzer 2100 (Agilent, Palo Alto, CA) to ensure that the ratio of 260 nm/280 nm exceeded 2.0, and that the ratio of 28S rRNA to 18S rRNA exceeded 1.0 for each sample. Pool 1 total RNA was used for the Affymetrix microarray

hybridization, and Pool 2 total RNA was used for validation of gene expression profiles by TaqMan® analysis.

[0398] Total RNA was also prepared, as described above, for Affymetrix GeneChip and TaqMan analysis from 27 rat organ tissues dissected from naïve rats.

- 5 These included duodenum, lung, ovary, esophagus, diaphragm, skin, heart, colon, optic nerve, thyroid, thymus, trachea, superior cervical ganglion, prostate, dorsal root ganglia, sciatic nerve, spinal cord, brain, adrenal, aorta, fetal brain, kidney, liver, quadriceps muscle, spleen, submaxillary gland, and testis.

6.3.2. Microarray Analysis

- 10 [0399] GeneChip® (Affymetrix, Santa Clara, CA) technology allows comparative analysis of the relative expression of thousands of known genes annotated in the public domain (herein, referred to as simply “known genes”), and genes encompassing ESTs (herein, referred to as simply “ESTs”), under multiple experimental conditions. Each gene is represented by a “probeset” consisting of
- 15 multiple pairs of oligonucleotides (25 nt in length) with sequence complementary to the gene sequence or EST sequence of interest, and the same oligonucleotide sequence with a one base-pair mismatch. These probeset pairs allow for the detection of gene-specific nucleic acid hybridization signals as described below. The Affymetrix Rat U34 A, B and C arrays used for the described analysis contain
- 20 probesets representing about 26,000 genes including 1200 genes of known relevance to the field of neurobiology. For example, these arrays include probesets specific for detecting the mRNA for kinases, cell surface receptors, cytokines, growth factors and oncogenes.

- [0400] Hybridization probes were prepared according to the Affymetrix
- 25 Technical Manual (available on the WorldWideWeb at affymetrix.com/support/technical/manual/expression_manual.affx). First-strand cDNA synthesis was primed for each total RNA sample (10 µg), using 5 mM of oligonucleotide primer encoding the T7 RNA polymerase promoter linked to oligo-dT₂₄ primer. cDNA synthesis reactions were carried out at 42°C using Superscript

II - reverse transcriptase (Invitrogen, Carlsbad, CA). Second-strand cDNA synthesis was carried out using DNA polymerase I and T4 DNA ligase. Each double-stranded cDNA sample was purified by sequential Phase Lock Gels (Brinkman Instrument, Westbury, NY) and extracted with a 1:1 mixture of phenol to chloroform (Ambion Inc., Austin, TX). Half of each cDNA sample was transcribed *in vitro* into copy RNA (cRNA) labeled with biotin-UTP and biotin-CTP using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochemicals, New York, NY). These cRNA transcripts were purified using RNeasy™ columns (Qiagen, Hilden Germany), and quantified by measuring absorption at 260nm/280nm. Aliquots (15 µg) of each cRNA sample were fragmented at 95°C for 35 min in 40 mM Tris-acetate, pH 8.0, 100 mM KOAc, and 30 mM MgOAc to a mean size of about 50 to 150 nucleotides. Hybridization buffer (0.1 M MES, pH 6.7, 1M NaCl, 0.01% Triton, 0.5 mg/ml BSA, 0.1 mg/ml H. sperm DNA, 50 pM control oligo B2, and 1x eukaryotic hybridization control (Affymetrix, Santa Clara, CA)) was added to each sample.

[0401] Samples were then hybridized to RG-U34 A, B, and C microarrays (Affymetrix) at 45°C for 16 h. Microarrays were washed and sequentially incubated with streptavidin phycoerythrin (Molecular Probes, Inc., Eugene, OR), biotinylated anti-streptavidin antibody (Vector Laboratories, Inc., Burlingame, CA), and streptavidin phycoerythrin on the Affymetrix Fluidic Station. Finally, the microarrays were scanned with a gene array scanner (Hewlett Packard Instruments, TX) to capture the fluorescence image of each hybridization. Microarray Suite 5.0 software (Affymetrix) was used to extract gene expression intensity signal from the scanned array images for each probeset under each experimental condition.

6.3.3. Statistical Criteria

[0402] Based on cumulative historical statistical analysis of replicate sample data (not shown), it was determined that the reproducibility of GeneChip data is dependent on the intensity of the signal. For intensities above 130, the reproducibility exhibits a coefficient of variation (CV; standard deviation divided by the average intensity) of 0.2 or better. Below 130, the reproducibility quickly falls

off to CVs approaching infinity. Therefore, for genes having a gene expression intensity greater than 130, there is a high confidence of greater than two standard deviations for apparent fold-changes of three-fold or more.

[0403] As has been observed by others (Wang *et al.*, *Neuroscience* 2002;
5 114: 529-546), the apparent gene regulation in L5 and L6 was much more robust than in L4 (data not shown). In order to optimize filtering criteria to reduce the about 26,000 rat genes represented on the GeneChip to those most relevant for pain, multiple filtering criteria were applied based on different threshold detection limits, and fold-regulation in various tissues and conditions. The best criteria that captured
10 the most genes known to be molecular substrates of pain, and most likely to be reproducibly regulated by the SNL model in L4, L5 or L6, are listed below.

[0404] For L4, it was required that:

1. The maximum value between L4 sham (ipsilateral), SNL (ipsilateral), and SNL (contralateral) be at least 130, AND
- 15 2. that the L4 SNL (ipsilateral) compared to L4 sham (ipsilateral) exhibit at least three-fold regulation, AND
3. that the L4 SNL (ipsilateral) compared to L4 SNL (contralateral) exhibit at least three-fold regulation.

[0405] For L5 and L6, it was required that:

- 20 1. The maximum value between L5 sham (ipsilateral), L5 SNL (ipsilateral), L6 sham (ipsilateral), and L6 SNL (ipsilateral) be at least 130, AND
2. that the L5 SNL (ipsilateral) compared to L5 sham (ipsilateral) exhibit at least three-fold regulation, AND
- 25 3. that the L6 SNL (ipsilateral) compared to L6 sham (ipsilateral) exhibit at least three-fold regulation.

[0406] Probesets representing 249 known genes and 87 ESTs were selected based on the above criteria. Thirteen genes known to be molecular mediators of pain captured by the filtering criteria included the vanilloid receptor (VR-1), voltage-gated sodium channels Na_v and SNS/PN3/Nav1.8, serotonin receptor (5HT₃), glutamate receptor (iGluR5), regulator of G protein signaling (RGS4), nicotinic acetylcholine receptor alpha 3 subunit, transcription factor DREAM, galanin receptor type 2, somatostatin, galanin, vasoactive intestinal peptide, and neuropeptide Y. Included among the ESTs was a DNA sequence annotated by Affymetrix as GenBank Accession No. AA891631 (SEQ ID NO: 10). **Figure 1A** provides the microchip signal intensities for EST AA891631 in L4 naïve ipsi, L4 naïve contra, L4 sham ipsi, L4 SNL ipsi, L4 SNL contra, L4 SNL+GPN ipsi, L5 naïve ipsi, L5 sham ipsi, L5 SNL ipsi, and L5 SNL+GPN ipsi samples. As shown in **Figure 1A**, EST AA891631 is downregulated in the L5 DRG of SNL animals by about 3-fold as compared to L5 DRG in naïve or sham-operated animals.

[0407] In order to further characterize the 336 genes (249 known plus 87 ESTs) regulated by SNL according to the stringent criteria described above, the present inventors used hierarchical clustering algorithms with a standard correlation distance measure available in GeneSpring software (Silicon Genetics, Redwood City, CA) to order the 336 genes based on their gene expression profiles. The experiment samples used for the hierarchical clustering analysis included: L4 naïve ipsi, L4 naïve contra, L4 sham ipsi, L4 SNL ipsi, L4 SNL contra, L4 GPN ipsi, L5 naïve ipsi, L5 sham ipsi, L5 SNL ipsi, L5 SNL contra, L5 SNL+GPN ipsi, L6 naïve ipsi, L6 sham ipsi, L6 SNL ipsi, L6 SNL contra, L6 SNL+GPN, sciatic nerve, spinal cord, brain, adrenal, aorta, fetal brain, kidney, liver, quadriceps muscle, spleen, submaxillary gland, and testis. Using the results of hierarchical clustering and determining the functional annotations of grouped genes, nine transcript regulation classes were determined and designated as: (1) known and novel DRG-specific pain targets; (2) neuronal cellular signal transduction proteins; (3) neuronal markers; (4) cellular signal transduction proteins; (5) known and novel neuropeptides or secreted molecules; (6) inflammatory response genes A; (7) inflammatory response genes B; (8) markers of muscle tissue; and (9) unknown.

The gene expression profile of EST AA891631 (SEQ ID NO: 10) is part of transcript class (1), consisting of probesets representing ten ESTs and twenty-two different known genes, including eight known pain genes (VR-1, NaN, SNS/PN3/Nav1.8, 5HT3, iGluR5, RGS4, nicotinic acetylcholine receptor, and DREAM).

- 5 The tight coupling of the EST AA891631 gene expression profile to that of multiple genes known to be molecular mediators of pain suggests that the full-length gene sequence encompassing EST AA891631 encodes a protein whose temporal and spatial expression and function is involved in pain.

10 **6.3.4 Tissue Expression Profile of EST AA891631 in Naïve Rat Tissue using Microarray Analysis**

- [0408] The expression profiles for EST AA891631 across 27 rat naïve tissue samples (duodenum, lung, ovary, esophagus, diaphragm, skin, heart, colon, optic nerve, thyroid, thymus, trachea, superior cervical ganglion, prostate, dorsal root ganglia, sciatic nerve, spinal cord, brain, adrenal, aorta, fetal brain, kidney, liver, 15 quadriceps muscle, spleen, submaxillary gland, and testis) was determined by Affymetrix GeneChip analysis, as described in Section 6.3.2.

- [0409] Results of the tissue expression analysis show that EST AA891631 is highly enriched in DRGs with an intensity of about 240 (**Figure 1B**), but is also 20 found in brain and spinal cord.

6.3.5. TaqMan® Quantitative Real-Time PCR

- [0410] The expression profiles across across 10 SNL samples (L4 naïve ipsi, L4 naïve contra, L4 sham ipsi, L4 SNL ipsi, L4 SNL contra, L4 SNL+GPN ipsi, 25 L5 naïve ipsi, L5 sham ipsi, L5 SNL ipsi, and L5 SNL+GPN ipsi) and 27 rat naïve tissue samples (duodenum, lung, ovary, esophagus, diaphragm, skin, heart, colon, optic nerve, thyroid, thymus, trachea, superior cervical ganglion, prostate, dorsal root ganglia, sciatic nerve, spinal cord, brain, adrenal, aorta, fetal brain, kidney, liver, quadriceps muscle, spleen, submaxillary gland, and testis) for 18 known genes

in transcript class 1 and for EST AA891631 (**Figures 1A-B**) were confirmed by TaqMan analysis as described below.

- [0411] Total RNA (10 ng, produced as described above) was used to synthesize cDNA with random hexamers using a TaqMan® Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed on an Applied Biosystems ABI Prism 7700 Sequence Detection System. Matching primers and fluorescence probes were designed for the gene or EST sequences using Primer Express software from Applied Biosystems. Primers and probe sequences used for rat EST AA891631 are listed in **Table 2**.

10

Table 2. List of nucleotide sequences (with nucleotide sequences shown from 5' to 3'):

SEQ ID NO	Primer Description	Sequence
14	Forward TaqMan primer for EST AA891631	TCCGGGTGTGTATGTGTCTTTG
15	Reverse TaqMan primer for EST AA891631	GGTTTGCCAGCCTGGTGTAC
16	TaqMan probe sequence for EST AA891631	TCTCTTTCTGTGTCTAGGACTCTTC
17	Sequence specific primer for EST AA891631, "MB0171", used for probe amplification in Northern Blot and <i>in situ</i> analysis	AATTAACCCTCACTAAAGGGAGCAGAGACT CAAGCCAGG
18	Sequence specific primer for EST AA891631, "MB0172", used for probe amplification in Northern Blot and <i>in situ</i> analysis	TAATACGACTCACTATAGGGTATCTCCCAG GAAGGAGAA

SEQ ID NO	Primer Description	Sequence
23	Sequence specific forward primer for SEQ ID NO: 20, "MB0436", used to amplify PNPG5 RTPCRa	TGCGATACCAAACAGCAAGGG
24	Sequence specific reverse primer for SEQ ID NO: 10, "MB0439", used to amplify PNPG5 RTPCRa	TCTCCAGAGGAACCTGCTTCTCCTTCC
25	Sequence specific forward primer for SEQ ID NO: 21, "MB0622", used to amplify PNPG5 RTPCRa	TCCCTGGCTGGGAGAAAGCC
26	Sequence specific reverse primer for SEQ ID NO: 21, "MB0623", used to amplify PNPG5 PTPCRb	TACTAATCAAGGCTTTCTCCCAGCC
27	Sequence specific reverse primer for SEQ ID NO: 21, "MB0737", used to amplify PNGP5 RTPCRb	AGCCAGGGAATTAGCCTACCTGG
28	Sequence specific forward primer for SEQ ID NO: 22, "MB0738", used to amplify PNGP5 RTPCRb	TGGGCTGAGCCGCATCCTCAC

SEQ ID NO	Primer Description	Sequence
29	Sequence specific forward primer for SEQ ID NO: 22, "MB0739", used to amplify PNGP5 RTPCRb	AGCAAGTTCCTCGCAGGCGATG

[0412] Both forward and reverse primers were used at 200 nM. In all cases, the final probe concentration was 200 nM. The real-time PCR reaction was performed in a final volume of 25 μ l using TaqMan® Universal PCR Master Mix containing AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs (with dUTP), Passive Reference 1, optimized buffer components (Applied Biosystems, Foster City, CA) and 5 μ l of cDNA template. Three replicates of reverse transcription and real-time PCR for each RNA sample were performed on the same reaction plate. A control lacking a DNA template, and controls using reference genes with stable expressions in all samples in the SNL/GPN study, were included on the same plate to minimize the reaction variability.

[0413] In quantitative real-time PCR, exponential amplification of the initial target cDNA is reflected by increasing fluorescence. The amplification cycle at which this measured fluorescence crosses a specified threshold determined by the experimenter to be in the log-linear phase of the amplification is called the cycle threshold or Ct value (according to the manual of the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA)). Assuming 100% efficiency of the exponential amplification, Ct values between samples can be directly compared with a difference of one Ct unit corresponding to a 2-fold difference in expression levels, two Ct units to 4-fold, three to 8-fold, and so on. For example in the table of Figure 1A, the average Ct for naïve L5 was 27.13, and for SNL (Chung) L5 was 29.24. Therefore, the apparent fold difference would be calculated to be $2^{(29.24-27.13)} = 4.3$ -fold. Alternatively, as a way to correct for dilution errors in pipetting independent samples, we can also compare the samples after

normalizing to a control gene (such as PITPNB in Figure 1A), whose expression level is known to be unaffected by the treatment. For example, by the latter calculation in Figure 1A, the fold difference between naïve and SNL after normalization to PITPNB is $1.22/0.22 = 5.5$ -fold.

5

[0414] In the SNL model, TaqMan data indicates that AA891631 was down-regulated within a range of 3.8 to 4.3-fold between L5 SNL and control (naïve or sham). (Figure 1A). Across naïve tissues, EST AA891631 is expressed highest in DRG with a relative gene expression level of 2.1-fold greater than the
10 next highest tissue, brain (Figure 1B). Relative to the average expression in non-neuronal tissues, EST AA891631 is expressed at least 8.3-fold greater.

6.3.6 Summary of Affymetrix GeneChip and TaqMan analysis of EST AA891631

[0415] By both Affymetrix GeneChip and TaqMan analysis, the expression
15 of EST AA891631 is decreased by about 4.3- or 3.8-fold in injured SNL L5 DRG compared to L5 DRG in naïve or sham-operated animals, respectively. EST AA891631 expression is also decreased but to a lesser extent in injured SNL L4 ipsi DRG (which is also known to experience pain) compared to L4 ipsi DRGs in naïve and sham-operated animals according to microchip analysis. By both Affymetrix
20 GeneChip and TaqMan analysis, EST AA891631 is highly enriched in rat DRGs, but is also found in brain and spinal cord. (Figure 1B).

6.4. Identification of PNPG5

[0416] *Northern Blot Analysis.* As a first step to identify the rat gene encompassing EST AA891631, Northern blot analysis was performed. Using two
25 EST sequence-specific primers, MB0171 and MB0172 (Table 2, SEQ ID NOS: 17 and 18, respectively), a 300 bp DNA fragment was amplified by PCR from an in house rat DRG cDNA library for probe synthesis. ³²P-dATP labeled probe (SEQ ID NO: 8) prepared by using a random primer labeling kit (Stratagene Primer-it II Random Primer Labeling Kit; Catalog# 300385; Stratagene, La Jolla, CA) was then

hybridized to a rat Multiple Tissue Northern Blot (Catalog No. Blot-1; Sigma, Saint Louis, MO). Hybridization was performed as described by the BD BioSciences Multiple Tissue Northern Blot User Manual (Catalog# PT1200-1; BD BioSciences, Palo Alto, CA) and imaged by exposure to Kodak BioMax MS scientific imaging film for 14 hours at -70°C. **Figure 2** shows a rat transcript of about 6 kb in length, which is highly enriched in brain tissue. Smaller molecular weight bands observed in the Northern likely represent nonspecific binding. The relative intensity of these smaller bands varies consistently in a sample dependent fashion as if they vary with the amount of RNA loaded. However, in this experiment, we cannot rule out that there may be additional isoforms of the gene corresponding to EST AA891631.

[0417] *Determination of the cDNA sequence encompassing EST AA891631.*

The following description details the determination of the rat, mouse, and human PNPG5 cDNA sequences (SEQ ID NOS: 1, 3, and 5, respectively) and their corresponding protein sequences (SEQ ID NOS: 2, 4, and 6, respectively).

[0418] In order to determine the gene represented by EST AA891631, we first identified public domain EST clones encompassing or overlapping the sequences of EST AA891631. The goal was to obtain plasmid material for such clones, submit the material to dideoxy-based sequencing analysis, and determine the sequence for the entire cDNA insert beyond the previously sequenced EST portion. Invitrogen Life Technologies is a private company that archives public domain ESTs from the I.M.A.G.E. consortium (available on the WorldWideWeb at image.llnl.gov/), and provides them to researchers on a fee for service basis. Clones of interest can be identified and ordered via an online BLAST analysis using CloneRanger™ (available on the WorldWideWeb at clones.invitrogen.com/cloneranger.php). By BLAST analysis of Invitrogen's I.M.A.G.E. consortium archive, public domain EST clone UI-R-CV0-brl-a-03-0-UI (about 1.6 kb in length, SEQ ID NO: 19) was determined to be contiguous with the sequence of EST AA891631. This clone was obtained from Invitrogen (Catalog No. 99002; Carlsbad, CA), and the sequence for the entire cDNA insert (beyond the publicly available EST end-reads) was finished by dideoxy-sequencing. The DNA sequencing reaction was carried out using the DTCS Quick Start Kit and sequenced

on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The finished sequence for EST clone UI-R-CV0-brl-a-03-0-UI (herein referred to as "Contig 1631a", SEQ ID NO: 19) was aligned in Sequencher™ (Gene Codes Corporation, Ann Arbor, MI) to EST AA891631 (SEQ ID NO: 10) showing that

5 Contig 1631a is contiguous to the 5' end of EST AA891631.

[0419] Whereas the sequence of EST AA891631 alone did not lead to the identification of additional upstream sequences, the finished sequence for EST clone UI-R-CV0-brl-a-03-0-UI (Contig 1631a; SEQ ID NO: 19) identified a high scoring human cDNA (GenBank Accession No. AB032989, SEQ ID NO: 36) by BLAST

10 comparison to the NCBI nr database (available on the WorldWideWeb at www.ncbi.nlm.nih.gov/BLAST/). AB032989 is a partial cDNA sequence with a predicted partial open reading frame for a protein (GenBank Accession No. BAA86477, SEQ ID NO: 37). BLAST comparison of the human cDNA (SEQ ID NO: 36) to the NCBI nr database identified a high scoring hit for a mouse RIKEN

15 cDNA (GenBank Accession No. AK035960, SEQ ID NO: 34) with a corresponding protein sequence having a GenBank Accession No. BAC29259 (SEQ ID NO: 35). A BLAST comparison of the partial human cDNA sequence (SEQ ID NO: 36) to public rat ESTs (available on the WorldWideWeb at www.ncbi.nlm.nih.gov/BLAST/) identified two high scoring EST sequences

20 (GenBank Accession No. CB546119, SEQ ID NO: 20 and GenBank Accession No. CB696950, SEQ ID NO: 21). Again by BLAST analysis, these two rat EST sequences are located upstream of EST AA891631 and map to the same region in the rat genome (GenBank Accession No. NW_047626, a contig sequence located on Chromosome 2, SEQ ID NO: 11), providing further evidence that they belong to

25 the same rat transcript. The alignment of the mouse cDNA (GenBank Accession No. AK035960; SEQ ID NO: 34) to the rat genome identified the same region.

[0420] This alignment of mouse cDNA to rat genomic sequence was used to predict the rat gene corresponding to EST AA891631 (referred to herein as "Genomic PNPG5", SEQ ID NO: 22). This sequence consists of base pairs

30 12127225 to 12132649 of the contig sequence with GenBank Accession No. NW_047626 located on Chromosome 2 of the rat genome. From this alignment, it

was determined that Genomic PNPG5 is devoid of introns and can therefore, also be considered as the predicted cDNA sequence for PNPG5. Seven RT-PCR primers were designed as shown in **Table 2** to confirm that Genomic PNPG5 (SEQ ID NO: 22) is also the cDNA sequence encoding the ortholog of the mouse cDNA sequence with GenBank Accession No. AK035960 (SEQ ID NO: 34).

[0421] Because the predicted Genomic PNPG5 sequence is about 5 kb in length, it was difficult to obtain the entire cDNA by a single RT-PCR amplification. Therefore, a strategy was developed to obtain two PCR products, PNPG5 RTPCRa and PNPG5 RTPCRb, covering most of the entire length of Genomic PNPG5. The first RT-PCR product (PNPG5 RTPCRa, 2.4 kb, SEQ ID NO: 30) covers the middle of predicted Genomic PNPG5 to about the 3' end, including a portion of the sequence of EST AA891631 that was differentially expressed during the SNL model of pain. The second RT-PCR product (PNPG5 RTPCRb, 2.8 kb, SEQ ID NO:31) includes the open reading frame (ORF) of Genomic PNPG5 and contains a 3' end that overlaps with the 5' end of PNPG5 RTPCRa. This open reading frame sequence is designated as SEQ ID NO: 7. The technique of nested RT-PCR was used as described below to amplify PNPG5 RTPCRa and PNPG5 RTPCRb.

[0422] Total RNA isolated from naïve rat DRGs, as described above, was used as a template for RT-PCR. Two rounds of PCR were performed. In the first round, PCR primers, MB436/MB439 (SEQ ID NOS: 23 and 24, respectively) and MB739/MB623 (SEQ ID NOS: 29 and 26, respectively) were used to amplify PNPG5 RTPCRa and b, respectively. For the 2nd round of nested PCR, primers MB622/439 (SEQ ID NOS: 25 and 24) and MB738/737 (SEQ ID NOS: 28 and 27) were used to amplify PNPG5 RTPCRa and b, respectively, from a 1:100 dilution of the template from the 1st round of PCR. As predicted, PNPG5 RTPCRa and b were 2.4 and 2.8 kb in length, respectively, according to agarose gel electrophoresis of the 2nd round PCR products. Each product was then sub-cloned into a pCR4-TOPO vector (Catalog No. K4575-J10, Invitrogen, Carlsbad, CA) and sequenced as described previously.

[0423] The sequences for PNPG5 RTPCRa and b (SEQ ID NOS: 30 and 31, respectively) align contiguously. The combined sequence of PNPG5 RTPCRa and b aligns with rat Genomic PNPG5 (SEQ ID NO: 22) and the human (SEQ ID NO: 36) and mouse ortholog sequences (SEQ ID NO: 34). **Figure 7** shows the alignment of these sequences by Sequencher (Gene Codes Corporation Ann Arbor MI) and their relationship with the original EST AA891631 (1631-affy) sequence (SEQ ID NO: 10). The sequences of PNPG5 RTPCRa, b, and EST AA891631 can be contiguously aligned to form a cDNA sequence designated here as SEQ ID NO: 32 and referred to herein as "PNPG5 CONTIGab". The predicted protein sequence (SEQ ID NO: 33) for PNPG5 CONTIGab was determined from the ORF of PNPG5 RTPCRb (SEQ ID NO: 31) according to its homology with the mouse RIKEN cDNA with GenBank Accession No. AK035960 (SEQ ID NO: 34) and its corresponding protein (GenBank Accession No: BAC29259, SEQ ID NO: 35). The corresponding amino acid sequence for the orthologous human protein has a GenBank Accession No. of BAA86477 (SEQ ID NO: 37).

[0424] Comparing the predicted Genomic PNPG5 sequence (SEQ ID NO: 22) and the sequenced PNPG5 RTPCRb (SEQ ID NO: 31), three base pair differences were found in the ORF. The differences are located at nucleotide positions 1062, 1091, and 1391 of SEQ ID NO: 31 where a C, A, and T in the predicted sequence are an A, G, and A in the PNPG5 RTPCRb sequence, respectively. No amino acid change occurs at position 1062. At position 1091, an Asn is converted to a Ser at amino acid 182 of SEQ ID NO: 2; and at position 1391, a Leu is converted to a Gln at amino acid 282 of SEQ ID NO: 2.

[0425] The PNPG5 CONTIGab sequence (SEQ ID NO: 32) was used in a BLAST search of the NCBI nr database to identify a rat open reading frame sequence encoding for a protein referred to as AMIGO in Kuja-Panula, *et al.* (*J. Cell Bio.* 2003, 160:963-73). The AMIGO sequence will be referred herein as rat PNPG5 (SEQ ID NO: 1, GenBank Accession No. AY237729). A similar BLAST analysis using the mouse and human orthologs of PNPG5 CONTIGab (SEQ ID NOS: 34 and 36, respectively) identified homologous mouse and human AMIGO

proteins that will be designated herein as mouse and human PNPG5 (SEQ ID NOS: 3 and 5 with GenBank Accession Nos: AY237008 and AY237007, respectively).

[0426] *Rat PNPG5 protein and its orthologs.* The rat and human PNPG5 proteins (GenBank Accession Nos. AY237729 and AY237007 with SEQ ID NO: 2 and 6, respectively) have 493 amino acids and the corresponding mouse ortholog (GenBank Accession No. AY237008, SEQ ID NO: 4) has 492 amino acids (**Figure 3**). The extra amino acid in the rat and human protein sequences is located at position 323 with an Asp insertion.

[0427] Rat PNPG5 protein (SEQ ID NO: 2) shares **95%** and **89%** amino acid sequence identity with mouse PNPG5 (SEQ ID NO: 4) and human PNPG5 (SEQ ID NO: 6), respectively. The alignment of rat PNPG5, human PNPG5, mouse PNPG5, PNPG5 CONTIGab (SEQ ID NO: 32), its mouse ortholog (SEQ ID NO: 34), and its human ortholog (SEQ ID NO:36) was obtained using AlignX, Vector NTI software (Invitrogen Life Science Software, Frederick, MD) as shown in **Figure 3**.

[0428] As previously described, the rat PNPG5 genomic sequence (SEQ ID NO: 11) consists of base pairs 1217225 to 12132649 from contig sequence NW_047626 located on Chromosome 2 of the rat genome. These base pairs contain a single exon, predicted by PNPG5 CONTIGab (SEQ ID NO: 32), and an additional 2 kb of regulatory sequence both upstream and downstream of the predicted 5' and 3' UTRs of PNPG5 CONTIGab. Mouse and human genomic DNA sequences for PNPG5, SEQ ID NOS: 12 (NT_039239: 9936110 to 9942751 bp) and 13 (NT_019273:1040749-1049316), respectively, were determined by aligning the mouse transcript AK035960 (SEQ. ID NO 34) and human transcript AY237007 (SEQ. ID NO 5) to the mouse (NT_039239) and human genomic contigs (NT_019273), respectively. These predicted gene sequences also include about 2 kb of sequence upstream and downstream of the corresponding mouse (SEQ ID NO: 34) and human (SEQ ID NO: 5) PNPG5 cDNAs, in order to encompass potential regulatory regions.

[0429] *Summary.* A gene PNPG5 was identified based on the EST AA891631. EST AA891631 is differentially regulated in DRG in the rat SNL model of neuropathic pain when compared to control DRG. This EST was down-regulated similarly to class 1 transcripts including known pain-related genes, such as the
5 vallinoid receptor (VR-1), voltage-gated sodium channels (NaN), and SNS/PN3/Nav1.8. Since these known genes, such as the vanilloid receptor and the voltage-gated sodium channel, are known drug targets for analgesics, the present inventors conclude that PNPG5 is also useful as a drug target for pain and related disease states.

10

6.5. Further Characterization of PNPG5

[0430] *Bioinformatic analysis.* Analysis of the rat PNPG5 protein structure was performed using the Simple Modular Architecture Research Tool, SMART (Schultz *et al.*, *Proc. Natl. Acad. Sci. USA* 1998; 95: 5857-5864; and Letunic *et al.*, *Nucleic Acids Res.* 2002; 30: 242-244) (Figure 4). This algorithm predicts one
15 Leucine rich repeat N-terminal domain (LRRNT), two Leucine-rich repeats, typical (most populated) subfamily (LRR_TYP), three leucine rich repeats (LRR), one Leucine rich repeat C-terminal domains (LRRCT), an immunoglobulin domain (IG) and one transmembrane domain. The leucine-rich repeat is a recently characterized structural motif used in molecular recognition processes as diverse as signal
20 transduction, cell adhesion, cell development, DNA repair and RNA processing. All proteins containing these repeats are thought to be involved in protein-protein interactions. Leucine-rich repeats (LRR) are often flanked by cysteine rich domains, the N-terminal domain (LRRNT) and the C-terminal domain (LRRCT). Detailed descriptions of these domains are available on the WorldWideWeb at smart.embl-
25 heidelberg.de/.

[0431] *In situ hybridization.* The present inventors confirmed that PNPG5 is expressed in naïve DRG neurons by in situ hybridization (Figure 5).

[0432] A PNPG5-specific ³⁵S-UTP labeled antisense RNA probe (SEQ ID NO: 9) was generated using T7 RNA polymerase from a PCR template. The PCR

template was generated from a rat DRG cDNA library using rat PNPG5-specific primers, MB0171 and MB0172 (see Table 2; SEQ ID NOS: 17 and 18), containing T7 and T3 RNA polymerase promoter sequences.

[0433] The *in situ* hybridization protocol was performed according to Frantz
5 *et al.* (J. Neuroscience 1994, 14: 5725) with the exception of the proteinase K step
which was omitted. DRG from Sprague Dawley rats (Taconic, Germantown, NJ)
were dissected and frozen in TBS Tissue Freezing Medium™ (Triangle Biomedical
Sciences, Durham, NC). Frozen sections (20 µm thick) were fixed with 4%
paraformaldehyde onto Fisher Scientific Superfrost glass slides (Pittsburgh, PA).
10 Tissue sections were washed with PBS, treated with 0.25% Acetic Anhydride in
0.1M Triethanolamine, and dehydrated using a series of four ethanol washes, (using
50%, 70%, and 2 times 95% ethanol in water).

[0434] Sections were incubated with 6×10^6 cpm/ml of ^{35}S -labeled RNA
probe in hybridization buffer (62.5% formamide, 12.5% Dextran sulfate, 0.0025%
15 polyvinylpyrrolidone, 0.0025% ficoll, 0.0025% bovine serum albumin, 375mM
NaCl, 12.5mM Tris pH=8, 1.3mM EDTA, 10mM dithiothreitol (DTT), 150 µg/ml
E. coli tRNA) at 60°C for 16 hours. Sections were then treated with 50 µg/ml
RNaseA in 10mM Tris/0.5M NaCl and, subsequently, washed through a series of 4
SSC (0.15 M sodium chloride, 0.15 M sodium citrate) washes containing 1mM
20 DTT (using 2X SSC buffer, 1X SSC buffer, 0.5X SSC buffer, and 0.1X SSC
buffer). A final wash in 0.1X SSC, 1mM DTT buffer was performed for 30 min at
65°C. Sections were then dehydrated through a series of 6 ethanol washes (using
50%, 70%, 95% ethanol in water, and 3 times using 100% ethanol), air-dried, and
dipped in Kodak NTB2 emulsion (Rochester, NY). Sections were exposed on slides
25 for 2 weeks. Slides were developed using Kodak D19 developer and Rapid Fix
(Rochester, NY).

[0435] After slides were developed, they were counterstained with
hematoxylin (Hematoxylin Stain Gill Formulation #2, Fisher Scientific, Fair Lawn,
NJ) and Eosin-Y (Lerner Laboratories, Pittsburgh, PA). Developed slides were
30 first washed in water 3 times for 5 minutes each time and stained in hematoxylin

(2g/L) for 2 minutes. Excess hematoxylin was washed from the sections with water until the water was clear. Slides were then rinsed in 70% ethanol with 0.1% Sodium Borate for 2 minutes. Slides were then washed in water for 2 minutes, stained with Eosin-Y(0.5%) for 2 minutes, washed in water for 2 minutes, and then rinsed
5 through a series of alcohol washes (50%, 70%, 80%, 95%, 100%, and Xylene 2 times) for 1 minute each. Finally, a cover slip was applied using Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI). As seen in **Figure 5**, expression of PNPG5 is in small and large neurons of the DRG.

6.6. Investigation of Interactions between PNPG2 and PNPG5

10 6.6.1. Investigation of Interactions by Immunoprecipitation

[0436] *Construction of PNPG2-V5 and PNPG5-HA.* PNPG2-V5 and PNPG5-HA were separately cloned into Invitrogen vector pcDNA6.2/V5/GW/D-TOPO (Cat# K2460-20). The ORFs for PNPG2 and PNPG5 were obtained by RT-PCR amplification from rat DRG RNA with primers MB639/640 and MB861/862,
15 respectively (SEQ ID NOS: 42, 43, 44, and 45). For PNPG5, the reverse primer MB862 (SEQ ID NO: 45) contains HA tag sequence and a stop codon; therefore, the resulting recombinant consists of a fusion to the HA tag (not the V5 tag in the vector). For PNPG2, the reverse primer MB640 (SEQ ID NO: 43) does not contain a stop codon; therefore, the resulting recombinant results in a fusion to the V5-tag
20 from the vector. Cloning was performed as described by the manufacturer (Invitrogen Cat# K2460-20). Briefly, the purified PCR fragments were incubated with pcDNA-TOPO vector at room temperature for 5 min and transformed into One Shot TOP10 chemically competent *E. Coli* (Invitrogen Cat# C4040-03). The correct clones were confirmed by sequencing.

25 [0437] *Detection of PNPG2-PNPG5 interaction by immunoprecipitation.* Plasmids pcDNA-PNPG2-V5 and pcDNA-PNPG5-HA were transfected into HEK-293 cells in various combinations as indicated in **Figure 8A and B**. Immunoprecipitation was performed using either anti-HA antibody (**Figure 8A**) or anti-V5 antibodies (**Figure 8B**). For immunoprecipitation experiments, cells were
30 rinsed twice in ice-cold phosphate-buffered saline and lysed in PLC buffer (50 mM

Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM NaPPi, 100 mM NaF and a mixture of protease inhibitors) (Tong *et al.*, *JBC*, 2003 278:6111-6119). Protein concentrations of cleared lysates were determined using a BCA protein assay (Pierce, Cat#23225). Proteins were immunoprecipitated for 1-2 h at 4°C, and beads were washed three times with the same buffer. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen Cat#LC2000). The membranes were incubated with TBST containing 5% skim milk to block non-specific antibody binding, and then immunoblotted as per standard protocols. Primary antibodies were detected with secondary antibodies labeled with horseradish peroxidase, followed by treatment with Enhanced Chemiluminescence (Pierce Cat# 37070).

[0438] **Figures 8A and B** represent Western blot analysis of the products of the immunoprecipitation experiments performed in HEK-293 cells transfected with PNPG5-HA encoding for the rat PNPG5 protein (SEQ ID NO: 2) fused to HA epitope-tag sequence and/or expression construct pPNPG2-V5 containing the rat PNPG2 ORF (SEQ ID NO: 41) fused to V5 epitope-tag sequence, as indicated above the loading lanes. Immunoprecipitation was performed using either anti-HA antibodies (**A**) or anti-V5 antibodies (**B**). The Western blot was probed using anti-V5 antibodies (right panel) and anti-HA antibodies (left panel). Co-precipitation of PNPG2 with PNPG5 is detectable in lane 4 in the right panel of **Figure 8A**. Co-precipitation of PNPG5 with PNPG2 is detectable in lane 4 in the left panel of **Figure 8B**. Controls indicate that anti-HA antibody did pull down some PNPG2-V5 (lane 2 in the right panel of **Figure 8A**). Anti-V5 antibody did not pull down PNPG5-HA protein (lane 3 in the left panel of **Figure 8B**) in the absence of the PNPG2-V5 plasmid. These results indicate that the PNPG2 GPCR is interacting with PNPG5 in transiently transfected cells. As these proteins contain LRRs, these repeats may be mediating these interactions. Indeed, as disclosed by Kuja-Panula *et al.* (*J. Cell Biol.* 2003, 160: 963-973), the LRR-containing proteins AMIGO, AMIGO2, and AMIGO3 demonstrate homophilic and heterophilic binding.

6.6.2. Investigation of Inter- and Intra-Cellular Interactions by Measuring Changes in Cellular cAMP Levels

[0439] *Cell transfection.* HEK-293 cells were spread 1×10^5 cells/well in a 24-well plate. After 24 hours, the cells were co-transfected with 100 ng pCRE.Luc (a vector with a CRE promoter driving luciferase enzyme expression, Stratagene Cat# 219076) combined with 100 ng of the test plasmid: pBluescript SK as a control, or a plamid containing cDNA for PNPG2 and/or a plamid containing cDNA for PNPG5. Invitrogen's lipofectamine Plus Reagent (Cat# 10964) was used to transfect all the cells. Briefly, 4 μ l of Plus Reagent (transfection agent; Cat.#4506, Ambion) was thoroughly mixed with 200 ng of DNA in Opti-MEM I medium to a final volume of 25 μ l in tube A, and incubated at room temperature (RT) for 15 minutes. 1 μ l of lipofactamine was mixed with 25 μ l of Opti-MEM I medium first in tube B and then added to tube A. The tube was incubated at room temperature for another 15 minutes, and spread to an individual well in a 24-well plate with 200 μ l of Opti-MEM I medium. After 3 hours, 1 ml of normal medium was added to each of 24 wells. After 24 hours, each well of cells from the 24-well plate was split into 6 wells of a 96-well plate for the measurement of luciferase activity in triplicate.

[0440] *Measurement of cAMP level by monitoring luciferase activity.* 48 hours after transfection, Bright-Glo™ Luciferase Assay System (Promega Cat. #E2610; Madison, WI) was used to measure luciferase activity. Briefly, the multi-well plates were removed from the incubator and allowed to equilibrate to room temperature. Then 100 μ l of substrate reagent from the kit was added to cells grown in 100 μ l of medium. The cells were incubated for 5 minutes at RT to allow complete cell lysis and luciferase activity was measured in a Wallace luminometer.

[0441] *Detection of PNPG5-PNPG2 interaction by measuring changes in cAMP levels.* Cellular cAMP levels were monitored by a DNA construct (pCRE-Luc, Stratagene Cat# 219076) containing the CRE (cAMP-responsive element) promoter driving luciferase gene expression. **Figure 9A** shows that when PNPG5 was co-transfected with PNPG2 in the same HEK-293 cell, a decrease in luciferase activity was observed suggesting that PNPG5 can interact with the GPCR, PNPG2, to reduce cAMP signaling. In **Figure 9B**, PNGP5 was transfected into HEK-293 cells separately and then mixed (in equal proportions) with an independent batch of

HEK-293 cells co-transfected with PNPG2 and the pCRE-Luc reporter. In the cell mixing experiment, PNPG5 appears to be capable of intercellular activation of PNPG2. Thus, co-transfection (**Figure 9A**) as well as intercellular mixing (**Figure 9B**) appear to be sufficient to induce a significant reduction in luciferase activity in
5 HEK-293 cells transfected with PNPG2 (and the pCRE-Luc reporter). These results suggest that PNPG5 can activate PNPG2-mediated reduction in cAMP levels through an activated G_i pathway.

6.7. Generation of Secreted Soluble PNPG5

[0442] The present prophetic example exemplifies a method for generating a
10 secreted soluble PNPG5 protein. A secreted soluble PNPG5 protein can be useful in the diagnostic, screening, and therapeutic methods as described above.

[0443] **Figure 10** shows a diagrammatic representation of the single transmembrane domain-containing protein PNPG5 compared to a recombinant form missing the transmembrane domain and fused with a His tag. This recombinant
15 protein can be produced as described below.

[0444] The vector expressing recombinant secreted soluble PNPG5 is constructed by PCR amplification of the PNPG5 extracellular domain from the vector containing PNPG5-HA which was constructed as described above. The PNPG5 extracellular domain is then subcloned into the pQE-TriSystem His-Strep 1
20 vector (Qiagen, Cat# 33913).

[0445] pQE-PNPG5N-His is packed into a linearized baculovirus (BacPac6 DNA) according to the standard protocol from BD Science Clontech (Cat# 6144-1). Briefly, Sf9 cells are spread onto a 6-well plate at 1.5×10^6 cells/well density, and allowed to attach to the well for 1 h. Cells are changed to 0.5 ml BacPAK Grace's
25 basic medium (#8091-1) for 30 min while the Bacfectin-DNA mixtures are prepared. 5 μ l of BacPAK6 DNA (Clontech, Cat# 6144-1) is mixed with 0.5 μ g of pQE-PNPG5N-His in 86 μ l of H₂O and 4 μ l of Bacfectin for 15 min. The Bacfectin-DNA mixture is added to the wells and the cells are incubated at 27°C for 5 hr. Then, 1.5 ml of BacPAK Complete Medium (#8090-1) is added to the wells

and 72 hr later, the medium, which contains virus produced by the transfected cells, is transferred to a sterile container and stored at 4°C. A plaque assay is performed with the cotransfection supernatant at dilutions of 10^{-1} , 10^{-2} and 10^{-3} to produce individual plaques. The plaque assay, recombinant identification and virus
5 purification are performed according to BD Pharmagen standard protocols (Cat# 554740).

[0446] After virus purification, the virus is amplified in 25 ml of medium in T175 flasks and the virus titer is determined. His-tag protein purification is carried out as described in the BD Pharmagen Baculovirus instruction manual (Cat#
10 554740). Sf9 cells in T150 flasks are infected with medium containing recombinant baculovirus (MOI=5) and incubated for 3 days. The supernatants from 20 flasks are collected and incubated with Ni-NTA agarose (Qiagen Cat# 1018244) at a concentration of 200 µl beads per 50 ml medium for 1 hr at 4°C. The beads are washed 3 times with 1 ml 6x His wash buffer (BD Biosciences Pharmingen, Cat#
15 554800) per 200 µl beads. His-fusion protein is eluted by adding equal volumes of 350 mM of imidazole in His elution buffer (BD Biosciences Pharmingen, Cat# 554800) to His beads for 3 min at room temperature. The elution solution is then dialyzed with Pierce's Slide-A-Dyzer Dialysis cassette (Cat# 66330) in PBS buffer. The final PNPG5N-His protein is separated by SDS-PAGE and detected by Simply
20 Blue SafeStain solution (Invitrogen, Cat# LC6060) and anti-His Antibody (Qiagen Cat# 27471001).

[0447] As indicated throughout the specification, a recombinant soluble PNPG5 produced as disclosed above can be useful for a number of research and therapeutic applications. For example, administration of a soluble PNPG5
25 polypeptide may have a therapeutic effect by interacting with cellular membrane-associated PNPG2 and reducing cAMP production (similar action to opioid agonists at opioid receptors).

7. Deposit of Biological Materials with the ATCC

[0448] The DNA product obtained by RT-PCR of naïve rat DRGs using primers MB738 and MB737 (SEQ ID NOS: 28 and 27, respectively) was subcloned into Invitrogen pCR4-TOPO vector (Cat# K4575-J10). The resulting plasmid
5 pPNPG5 comprising a polynucleotide molecule having a cDNA sequence encoding the rat PNPG5 protein was deposited with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, VA 20110-2209, USA on February 9, 2004, and has been assigned ATCC Accession No. PTA-5808.

* * *

10 [0449] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

15 [0450] All references cited herein, including all patents, published patent applications, and published scientific articles, are incorporated by reference in their entireties for all purposes. References include U.S. Provisional Application Serial Number 60/647,642, filed January 26, 2005, entitled "Pain-associated Gene PNPG6."

WHAT IS CLAIMED IS:

1. A genetically modified mammal that comprises cells that have been genetically modified so that a normal expression of a PNPG5-encoding gene has been reduced, eliminated, or increased.
- 5 2. A method for producing a genetically modified mammal, comprising genetically modifying one or more cells of said mammal such that the expression of the PNPG5 gene in said mammal has been modified compared to the expression of the PNPG5 gene in a wild-type mammal.
- 10 3. The method of claim 2, wherein said genetically modified cells are embryonic stem (ES) cells and wherein the intact genetically modified mammal is prepared utilizing said ES cells.
4. The method of claim 2, wherein said mammal is a mouse.
5. The method of claim 2, wherein the expression of the PNPG5 gene has been reduced or eliminated in at least some cells of said genetically modified
15 mammal.
6. The method of claim 2, wherein the expression of the PNPG5 gene has been increased in at least some cells of said genetically modified mammal.
7. An antisense oligonucleotide molecule that can specifically inhibit expression of a mammalian PNPG5 gene.
- 20 8. A ribozyme molecule that can specifically inhibit expression of a mammalian PNPG5 gene.
9. An interfering RNA molecule that can specifically inhibit expression of a mammalian PNPG5 gene.

10. A method for detecting a pain response in a test cell subjected to a treatment or stimulus or suspected of having been subjected to a treatment or stimulus, said method comprising:

- 5 (a) determining the expression level in the test cell of a nucleic acid molecule encoding a PNPG5 protein; and
- (b) comparing the expression level of the PNPG5-encoding nucleic acid molecule in the test cell to the expression level of the same nucleic acid molecule in a control cell not subjected to a treatment or stimulus;

10 wherein a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule in the test cell compared to the expression level of the PNPG5-encoding nucleic acid molecule in the control cell indicates that the test cell is exhibiting a pain response.

11. The method of claim 10, wherein the test and control cells naturally
15 express a PNPG5-encoding nucleic acid molecule.

12. The method of claim 10, wherein the test and control cells have been genetically modified to express a PNPG5-encoding nucleic acid molecule.

13. The method of claim 10, wherein the test and control cells are both from the central nervous system.

20 14. The method of claim 10, wherein the test and control cells are both from the peripheral nervous system.

15. The method of claim 10, wherein the test and control cells are both from the dorsal root ganglion (DRG).

25 16. The method of claim 10, wherein the test and control cells are neuronal cells.

17. The method of claim 10, wherein PNPG5-encoding nucleic acid encodes a rat, mouse or human PNPG5 protein.

18. The method of claim 17, wherein the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2; the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO:4; and the human PNPG protein comprises the amino acid sequence of SEQ ID NO:6.

19. The method of claim 10, wherein the expression level of the nucleic acid molecule in each of the test and control cells is determined by quantifying the amount of PNPG5-encoding mRNA present in the two cells.

20. The method of claim 10, wherein the expression level of the nucleic acid molecule in each of the test and control cells is determined by quantifying the amount of PNPG5 protein produced in each of the two cells.

21. A method for detecting a pain response in a test cell, said method comprising:

(a) determining in a test capable of expressing a PNPG5 protein the expression level of the PNPG5 protein; and

(b) comparing the expression level of the PNPG5 protein in the test cell to the expression level of the PNPG5 protein in a control cell not exhibiting a pain response;

wherein a detectable difference between the expression level of the PNPG5 protein in the test cell and the expression level of the PNPG5 protein in the control cell indicates that the test cell is exhibiting a pain response.

22. The method of claim 21, wherein the test cell is a neuronal cell.

23. The method of claim 21, wherein the PNPG5 protein is a rat, mouse or human PNPG5 protein.

24. The method of claim 23, wherein the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 2; the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 4; and the human PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 6.
- 5 25. The method of claim 21, wherein the expression level of the PNPG5 protein in the test cell is compared to the expression level of the PNPG5 protein in the neurons of the dorsal root ganglion (DRG).
26. The method of claim 21, wherein the amount of PNPG5 protein is determined by an immunoassay for PNPG5 protein.
- 10 27. The method of claim 21, wherein the test and control cells are from a mammal.
28. The method of claim 27, wherein the test and control cells are from a rat.
29. The method of claim 27, wherein the test and control cells are from a
15 mouse.
30. The method of claim 27, wherein the test and control cells are from a human.
31. A method for detecting a pain response in a test cell, said method comprising:
- 20 (a) determining in a test cell capable of expressing a PNPG5 protein an activity level of the PNPG5 protein; and
- (b) comparing the activity level of the PNPG5 protein in the test cell to the activity level of the PNPG5 protein in a control cell not exhibiting a pain response;

wherein a detectable difference between the activity level of the PNPG5 protein in the test cell and the activity level of the PNPG5 protein in the control cell indicates that the test cell is exhibiting a pain response.

32. The method of claim 31, wherein the activity level of the PNPG5
5 protein is determined by the level of its binding to a PNPG2 protein.

33. The method of claim 32, wherein the level of PNPG5 binding to a PNPG2 protein is determined using a method selected from the group consisting of immunoprecipitation, bioluminescence resonance energy transfer (BRET), 2-hybrid system, and BIACORE method.

10 34. The method of claim 31, wherein the activity level of the PNPG5 protein is determined by measuring the level of a G-protein coupled receptor (GPCR) activity of a PNPG2 protein.

35. The method of claim 34, wherein the GPCR activity of PNPG2 is measured by detecting a change in cAMP levels in a cell or detecting a change in
15 calcium levels in a cell or detecting redistribution of an arrestin molecule from the cytoplasm to the plasma membrane.

36. The method of claim 34, wherein the GPCR activity of PNPG2 is measured by detecting a change in cAMP levels in a cell.

37. The method of claim 36, wherein the change in cAMP levels is
20 measured by the effect on CRE-mediated luciferase activity.

38. The method of claim 32 or 34, wherein the PNPG5 protein and the PNPG2 protein are present on the same cell.

39. The method of claim 32 or 34, wherein the PNPG5 protein and the PNPG2 protein are present on different cells.

25 40. The method of claim 31, wherein the test cell is a neuronal cell.

41. The method of claim 31, wherein the PNPG5 protein is a rat, mouse or human PNPG5 protein.

42. The method of claim 41, wherein the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 2; the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 4; and the human PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 6.

43. The method of claim 31, wherein the activity level of the PNPG5 protein in the test cell is compared to the activity level of the PNPG5 protein in the neurons of the dorsal root ganglion (DRG).

44. The method of claim 31, wherein the test and control cells are from a mammal.

45. The method of claim 44, wherein the test and control cells are from a rat.

46. The method of claim 44, wherein the test and control cells are from a mouse.

47. The method of claim 44, wherein the test and control cells are from a human.

48. A method for identifying a candidate compound useful for modulating the expression of a PNPG5-encoding nucleic acid, said method comprising:

(a) contacting a first cell with a test compound under conditions sufficient to allow the cell to respond to said contact with the test compound;

(b) determining in the cell prepared in step (a) the expression level of a PNPG5-encoding nucleic acid molecule; and

(c) comparing the expression level of the PNPG5-encoding nucleic acid molecule determined in step (b) to the expression

level of the PNPG5-encoding nucleic acid molecule in a second (control) cell that has not been contacted with the test compound;

wherein a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule in the first cell in response to contact with the test compound
5 compared to the expression level of the PNPG5-encoding nucleic acid molecule in the second cell that has not been contacted with the test compound, indicates that the test compound modulates the expression of the PNPG5-encoding nucleic acid and is a candidate compound.

49. The method of claim 48, wherein the test compound decreases the
10 expression of the PNPG5-encoding nucleic acid molecule.

50. The method of claim 48, wherein the test compound increases the expression of the PNPG5-encoding nucleic acid molecule.

51. The method of claim 48, wherein the test compound is a small inorganic molecule, a small organic molecule, a polypeptide, a nucleic acid
15 molecule, or a chimera or derivative thereof.

52. The method of claim 48, wherein the cells used constitutively express a PNPG5-encoding nucleic acid molecule.

53. The method of claim 48, wherein the cells used express a PNPG5-encoding nucleic acid molecule in response to a specific stimulus.

20 54. The method of claim 48, wherein the cells used have been genetically modified to express a PNPG5-encoding nucleic acid molecule.

55. The method of claim 48, wherein the cells used are from the central nervous system.

25 56. The method of claim 48, wherein the cells used are from the peripheral nervous system.

57. The method of claim 48, wherein the cells used are from the dorsal root ganglion (DRG).

58. The method of claim 48, wherein the cells used are neuronal cells.

59. The method of claim 48, wherein the PNPG5-encoding nucleic acid
5 encodes a mammalian PNPG5 protein.

60. The method of claim 59, wherein the PNPG5-encoding nucleic acid encodes a rat, mouse or human PNPG5 protein.

61. The method of claim 60, wherein the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2; the mouse PNPG5 protein comprises the
10 amino acid sequence of SEQ ID NO:4; and the human PNPG protein comprises the amino acid sequence of SEQ ID NO:6.

62. The method of claim 48, wherein the cells used are from an animal model of pain.

63. The method of claim 48, wherein the cells used are from a human or
15 companion animal subject.

64. The method of claim 48, wherein the expression level of the PNPG5-encoding nucleic acid molecule is determined by quantifying the amount of PNPG5-encoding mRNA present in the cells.

65. The method of claim 48, wherein the expression level of the PNPG5-
20 encoding nucleic acid molecule is determined by quantifying the amount of PNPG5 protein present in the cells.

66. A method for identifying a candidate compound useful for modulating the expression of a PNPG5 protein, said method comprising:

(a) contacting a first cell with a test compound under conditions
25 sufficient to allow the cell to respond to said contact with the test compound;

(b) determining in the cell prepared in step (a) the expression level of a PNPG5 protein; and

5 (c) comparing the expression level of the PNPG5 protein determined in step (b) to the expression level of the PNPG5 protein in a second (control) cell that has not been contacted with the test compound;

wherein a detectable change in the expression level of the PNPG5 protein in the first cell in response to contact with the test compound compared to the expression level of the PNPG5 protein in the second cell that has not been contacted with the test
10 compound, indicates that the test compound modulates the expression of the PNPG5 protein and is a candidate compound.

67. The method of claim 66, wherein the test compound decreases the expression of the PNPG5 protein.

68. The method of claim 66, wherein the test compound increases the
15 expression of the PNPG5 protein.

69. The method of claim 66, wherein the test compound is a small inorganic molecule, a small organic molecule, a polypeptide, a nucleic acid molecule, or a chimera or derivative thereof.

70. The method of claim 66, wherein the cells used constitutively express
20 a PNPG5 protein.

71. The method of claim 66, wherein the cells used express a PNPG5 protein in response to a specific stimulus.

72. The method of claim 66, wherein the cells used have been genetically modified to express a PNPG5 protein.

25 73. The method of claim 66, wherein the cells used are from the central nervous system.

74. The method of claim 66, wherein the cells used are from the peripheral nervous system.

75. The method of claim 66, wherein the cells used are from the dorsal root ganglion (DRG).

5 76. The method of claim 66, wherein the cells used are neuronal cells.

77. The method of claim 66, wherein the PNPG5 protein is a mammalian PNPG5 protein.

78. The method of claim 77, wherein the PNPG5 protein is a rat, mouse or human PNPG5 protein.

10 79. The method of claim 78, wherein the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2; the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO:4; and the human PNPG protein comprises the amino acid sequence of SEQ ID NO:6.

15 80. The method of claim 66, wherein the cells used are from an animal model of pain.

81. The method of claim 66, wherein the cells used are from a human or companion animal subject.

20 82. The method of claim 66, wherein the expression level of the PNPG5 protein is determined using antibodies that specifically bind to an isolated polypeptide comprising of the amino acid sequence of a mammalian PNPG5 protein.

83. A method for identifying a compound useful for modulating an activity of a PNPG5 protein, said method comprising:

25 (a) contacting a PNPG5 protein with a test compound under conditions sufficient to allow the protein to respond to said contact with the test compound;

- (b) determining the level of an activity of the PNPG5 protein during or after contact with the test compound; and
- (c) comparing the activity of the PNPG5 protein determined in step (b) to the activity of the PNPG5 protein when it is not in contact with the test compound;

5

wherein a detectable difference between the activity of the PNPG5 protein in response to contact with the test compound and the activity of the PNPG5 protein when it is not in contact with the test compound indicates that the test compound modulates the activity of the PNPG5 protein.

10 84. The method of claim 83, wherein the activity level of the PNPG5 protein is determined by the level of its binding to a PNPG2 protein.

 85. The method of claim 84, wherein the level of PNPG5 binding to a PNPG2 protein is determined using a method selected from the group consisting of immunoprecipitation, bioluminescence resonance energy transfer (BRET), 2-hybrid
15 system, and BIACORE method.

 86. The method of claim 83, wherein the activity level of the PNPG5 protein is determined by measuring the level a G-protein coupled receptor (GPCR) activity of a PNPG2 protein.

 87. The method of claim 86, wherein the GPCR activity of PNPG2 is
20 measured by detecting a change in cAMP levels in a cell or detecting a change in calcium levels in a cell or detecting redistribution of an arrestin molecule from the cytoplasm to the plasma membrane.

 88. The method of claim 86, wherein the GPCR activity of PNPG2 is measured by detecting a change in cAMP levels in a cell.

25 89. The method of claim 88, wherein the change in cAMP levels is measured by the effect on CRE-mediated luciferase activity.

90. The method of claim 84 or 86, wherein the PNPG5 protein and the PNPG2 protein are present on the same cell.

91. The method of claim 84 or 86, wherein the PNPG5 protein and the PNPG2 protein are present on different cells.

5 92. The method of claim 83, wherein the test compound decreases the activity of the PNPG5 protein.

93. The method of claim 83, wherein the test compound increases the activity of the PNPG5 protein.

10 94. The method of claim 83, wherein the test compound is a small inorganic molecule, a small organic molecule, a polypeptide, a nucleic acid molecule, or a chimera or derivative thereof.

15 95. The method of claim 83, wherein the PNPG5 protein contacted with the test protein and the PNPG5 protein not contacted with the test protein are expressed by a first cell and second cell, respectively, that constitutively express the PNPG5 protein.

96. The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that express the PNPG5 protein in response to a specific stimulus.

20 97. The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that have been genetically modified to express or overexpress the PNPG5 protein.

25 98. The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that are neuronal cells.

99. The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that are from the central nervous system.

5 100. The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that are from the peripheral nervous system.

10 101. The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that are from the dorsal root ganglion (DRG).

 102. The method of claim 101, wherein the first cell and second cell are neurons of the DRG.

15 103. The method of claim 83, wherein the PNPG5 protein is a mammalian PNPG5 protein.

 104. The method of claim 103, wherein the PNPG5 protein is a rat, mouse or human PNPG5 protein.

20 105. The method of claim 104, wherein the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 2; the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 4; and the human PNPG5 protein comprises the amino acid sequence of SEQ ID NO: 6.

25 106. The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that are from an animal model of pain or related disorder.

The method of claim 83, wherein the PNPG5 protein contacted with the test compound and the PNPG5 protein not contacted with the test compound are expressed by a first cell and second cell, respectively, that are from a human or companion animal subject.

5 107. A method for monitoring the efficacy of an analgesic treatment in a cell comprising:

- (a) administering to said cell an analgesic compound under conditions sufficient to allow the cell to respond to said compound;
- (b) determining in the cell prepared in step (a) the expression
10 level of a PNPG5-encoding nucleic acid molecule; and
- (c) comparing the expression level of the PNPG5-encoding
 nucleic acid molecule determined in step (b) to the expression
 level of the PNPG5-encoding nucleic acid molecule in a second
 (control) cell that has not been contacted with the analgesic
15 compound;

wherein a detectable change in the expression level of the PNPG5-encoding nucleic acid molecule in the first cell in response to contact with the analgesic compound compared to the expression level of the PNPG5-encoding nucleic acid molecule in the second (control) cell that has not been contacted with the analgesic compound is
20 indicative of the activity of the analgesic compound.

108. The method of claim 107, wherein the cells used are from the central nervous system.

109. The method of claim 107, wherein the cells used are from the peripheral nervous system.

25 110. The method of claim 107, wherein the cells used are from the dorsal root ganglion (DRG).

111. The method of claim 107, wherein the cells used are neuronal cells.

112. The method of claim 107, wherein the cells used are from an animal model of pain.

113. The method of claim 107, wherein the cells used are from a human or
5 companion animal subject.

114. The method of claim 107, wherein the expression level of the PNPG5-encoding nucleic acid molecule is determined by quantifying the amount of PNPG5-encoding mRNA present in the cells.

115. The method of claim 107, wherein the expression level of the
10 PNPG5-encoding nucleic acid molecule is determined by quantifying the amount of PNPG5 protein present in the cells.

116. A method for identifying a candidate compound capable of binding to a PNPG5 protein, said method comprising:

15 (a) contacting a PNPG5 protein with a test compound under conditions that permit binding of the test compound to the PNPG5 protein; and

(b) detecting binding of the test compound to the PNPG5 protein.

117. The method of claim 116 wherein the test compound is a small inorganic molecule, small organic molecule, a polypeptide, a nucleic acid molecule,
20 or a chimera or derivative thereof.

118. The method of claim 116, wherein the PNPG5 protein is: (a) present in an intact cell that expresses the PNPG5 protein; (ii) present in a membrane fraction comprising the PNPG5 protein; or (iii) is an isolated PNPG5 protein that is unassociated with a cell or membrane fraction.

119. The method of claim 116, wherein the PNPG5 protein is produced in a cell that constitutively expresses a PNPG5-encoding nucleic acid molecule.

120. The method of claim 116, wherein the PNPG5 protein is produced in a cell that expresses a PNPG5-encoding nucleic acid molecule in response to a
5 specific stimulus.

121. The method of claim 116, wherein the PNPG5 protein is produced in a cell that has been genetically modified to express or overexpress a PNPG5-encoding nucleic acid molecule.

122. The method of claim 116, wherein the PNPG5 protein is produced in
10 a cell from the central nervous system.

123. The method of claim 116, wherein the PNPG5 protein is produced in a cell from the peripheral nervous system.

124. The method of claim 116, wherein the PNPG5 protein is produced in a cell from the dorsal root ganglion (DRG).

125. The method of claim 116, wherein the PNPG5 protein is produced in
15 a neuronal cell.

126. The method of claim 116, wherein the PNPG5 protein is a mammalian PNPG5 protein.

127. The method of claim 126, wherein the mammalian PNPG5 protein is
20 a rat, mouse or human PNPG5 protein.

128. The method of claim 127, wherein the rat PNPG5 protein comprises the amino acid sequence of SEQ ID NO:2; the mouse PNPG5 protein comprises the amino acid sequence of SEQ ID NO:4; and the human PNPG protein comprises the amino acid sequence of SEQ ID NO:6.

129. The method of claim 118, wherein the intact cell used is from an animal model of pain.

130. The method of claim 118, wherein the intact cell used is from a human or companion animal subject.

5 131. A method for identifying a compound capable of modulating binding between a PNPG5 protein and a PNPG2 protein, said method comprising determining the ability of the PNPG5 protein and the PNPG2 protein to bind together in the presence and absence of a test compound.

132. The method of claim 131, comprising:

10 (a) combining together a PNPG5 protein and a PNPG2 protein in the presence of a test compound under conditions that would permit binding of the PNPG5 protein to the PNPG2 protein in the absence of the test compound;

15 (b) determining the degree of binding of the PNPG5 protein to the PNPG2 protein in the presence of the test compound; and

 (c) comparing the degree of binding of the PNPG5 protein to the PNPG2 protein in the presence of the test compound with the degree of binding of the PNPG5 protein to the PNPG2 protein in the absence of the test compound;

20 wherein a detectable difference between the amount of the PNPG5 protein binding to the PNPG2 protein in the presence of the test compound and the amount of the PNPG5 protein binding to the PNPG2 protein in the absence of the test compound indicates that the test compound modulates binding between the PNPG5 protein and the PNPG2 protein.

25 133. A method for treating a condition that can be treated by modulating expression of a PNPG5-encoding nucleic acid molecule or a PNPG5 protein, comprising administering to a subject in need of such treatment a therapeutically

effective amount of a compound that modulates expression of a PNPG5-encoding nucleic acid molecule or a PNPG5 protein.

134. The method of claim 133, wherein the compound decreases expression of a PNPG5-encoding nucleic acid molecule or a PNPG5 protein.

5 135. The method of claim 133, wherein the compound increases expression of a PNPG5-encoding nucleic acid molecule or a PNPG5 protein.

136. The method of claim 133, wherein the treated condition is a pain or a related disorder.

10 137. The method of claim 133, wherein the treated condition is selected from the group consisting of neuropathic pain, nociceptive pain, chronic pain, inflammatory pain, pain associated with cancer, and pain associated with rheumatic disease.

15 138. The method of claim 133, wherein the treated condition is selected from the group consisting of addiction, seizure, stroke, ischemia, a neurodegenerative disorder, anxiety, depression, headache, asthma, rheumatic disease, osteoarthritis, retinopathy, inflammatory eye disorders, pruritis, ulcer, gastric lesions, uncontrollable urination, an inflammatory or unstable bladder disorders, inflammatory bowel disease, irritable bowel syndrome (IBS), irritable bowel disease (IBD), gastroesophageal reflux disease (GERD), functional dyspepsia, 20 functional chest pain of presumed oesophageal origin, functional dysphagia, non-cardiac chest pain, symptomatic gastroesophageal disease, gastritis, aerophagia, functional constipation, functional diarrhea, burbulence, chronic functional abdominal pain, recurrent abdominal pain (RAP), functional abdominal bloating, functional biliary pain, functional incontinence, functional ano-rectal pain, chronic 25 pelvic pain, pelvic floor dyssnergia, unspecified functional ano-rectal disorder, cholecystalgia, interstitial cystitis, dysmenorrhea, and dyspareunia.

139. A method for treating a condition that can be treated by modulating an activity of a PNPG5 protein, comprising administering to a subject in need of

such treatment a therapeutically effective amount of a compound that modulates the activity of the PNPG5 protein.

140. The method of claim 139, wherein the treated condition is a pain or a related disorder.

5 141. The method of claim 139, wherein the treated condition is selected from the group consisting of neuropathic pain, nociceptive pain, chronic pain, inflammatory pain, pain associated with cancer, and pain associated with rheumatic disease.

10 142. The method of claim 139, wherein the treated condition is selected from the group consisting of addiction, seizure, stroke, ischemia, a neurodegenerative disorder, anxiety, depression, headache, asthma, rheumatic disease, osteoarthritis, retinopathy, inflammatory eye disorders, pruritis, ulcer, gastric lesions, uncontrollable urination, an inflammatory or unstable bladder disorders, inflammatory bowel disease, irritable bowel syndrome (IBS), irritable
15 bowel disease (IBD), gastroesophageal reflux disease (GERD), functional dyspepsia, functional chest pain of presumed oesophageal origin, functional dysphagia, non-cardiac chest pain, symptomatic gastroesophageal disease, gastritis, aerophagia, functional constipation, functional diarrhea, burbulence, chronic functional abdominal pain, recurrent abdominal pain (RAP), functional abdominal bloating,
20 functional biliary pain, functional incontinence, functional ano-rectal pain, chronic pelvic pain, pelvic floor dyssnergia, unspecified functional ano-rectal disorder, cholecystalgia, interstitial cystitis, dysmenorrhea, and dyspareunia.

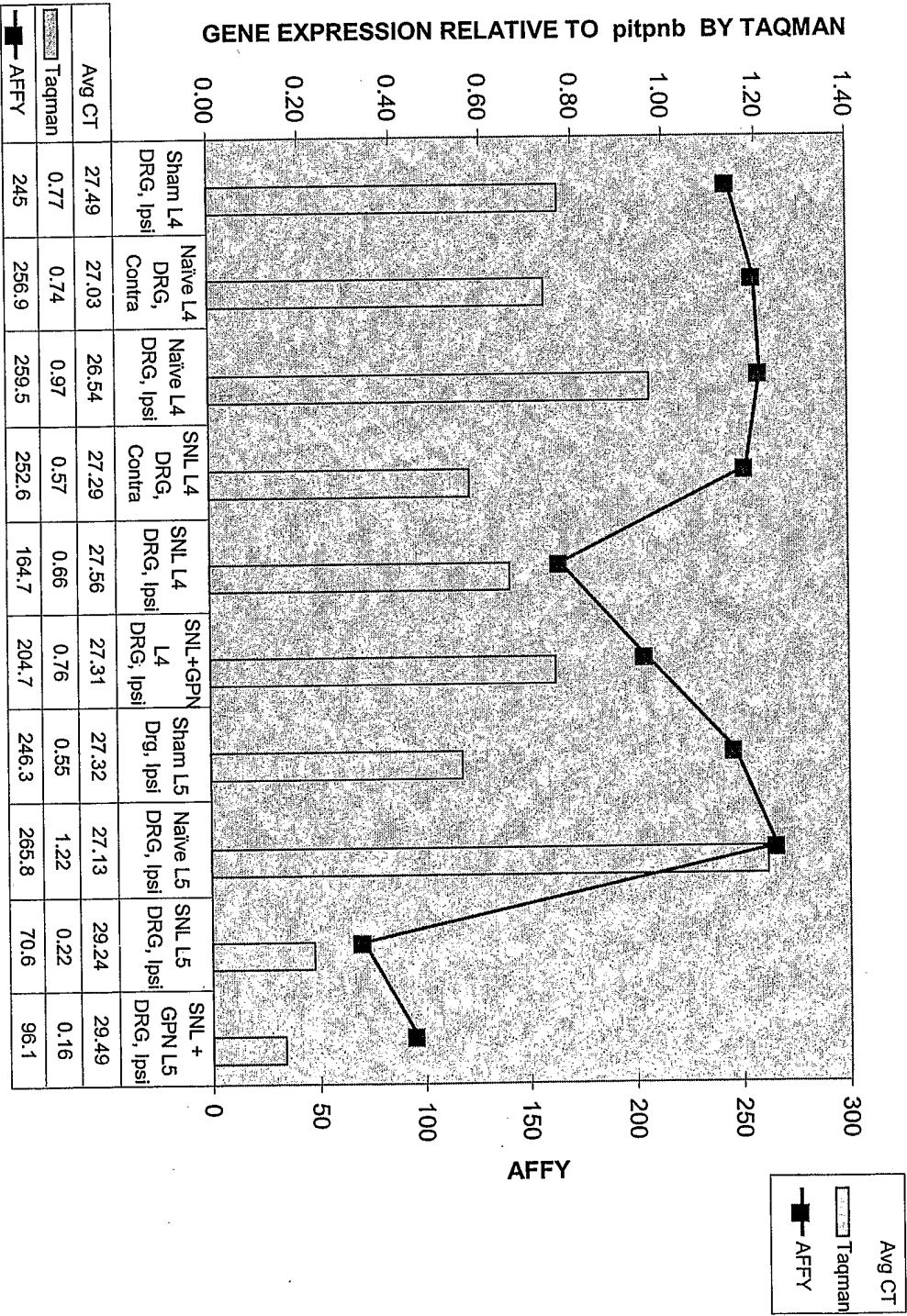
143. A method for treating a condition that can be treated by modulating the binding between a PNPG2 protein and a protein selected from the group
25 consisting of PNPG2, PNPG5, PNPG5 and PNPG3/AMIGO3, comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound that modulates such binding.

144. The method of claim 143, wherein the treated condition is a pain or a related disorder.

145. The method of claim 143, wherein the treated condition is selected from the group consisting of neuropathic pain, nociceptive pain, chronic pain, inflammatory pain, pain associated with cancer, and pain associated with rheumatic disease.

5 146. The method of claim 143, wherein the treated condition is selected from the group consisting of addiction, seizure, stroke, ischemia, a neurodegenerative disorder, anxiety, depression, headache, asthma, rheumatic disease, osteoarthritis, retinopathy, inflammatory eye disorders, pruritis, ulcer, gastric lesions, uncontrollable urination, an inflammatory or unstable bladder disorders, inflammatory bowel disease, irritable bowel syndrome (IBS), irritable
10 bowel disease (IBD), gastroesophageal reflux disease (GERD), functional dyspepsia, functional chest pain of presumed oesophageal origin, functional dysphagia, non-cardiac chest pain, symptomatic gastroesophageal disease, gastritis, aerophagia, functional constipation, functional diarrhea, burbulence, chronic functional
15 abdominal pain, recurrent abdominal pain (RAP), functional abdominal bloating, functional biliary pain, functional incontinence, functional ano-rectal pain, chronic pelvic pain, pelvic floor dyssnergia, unspecified functional ano-rectal disorder, cholecystalgia, interstitial cystitis, dysmenorrhea, and dyspareunia.

Figure 1A



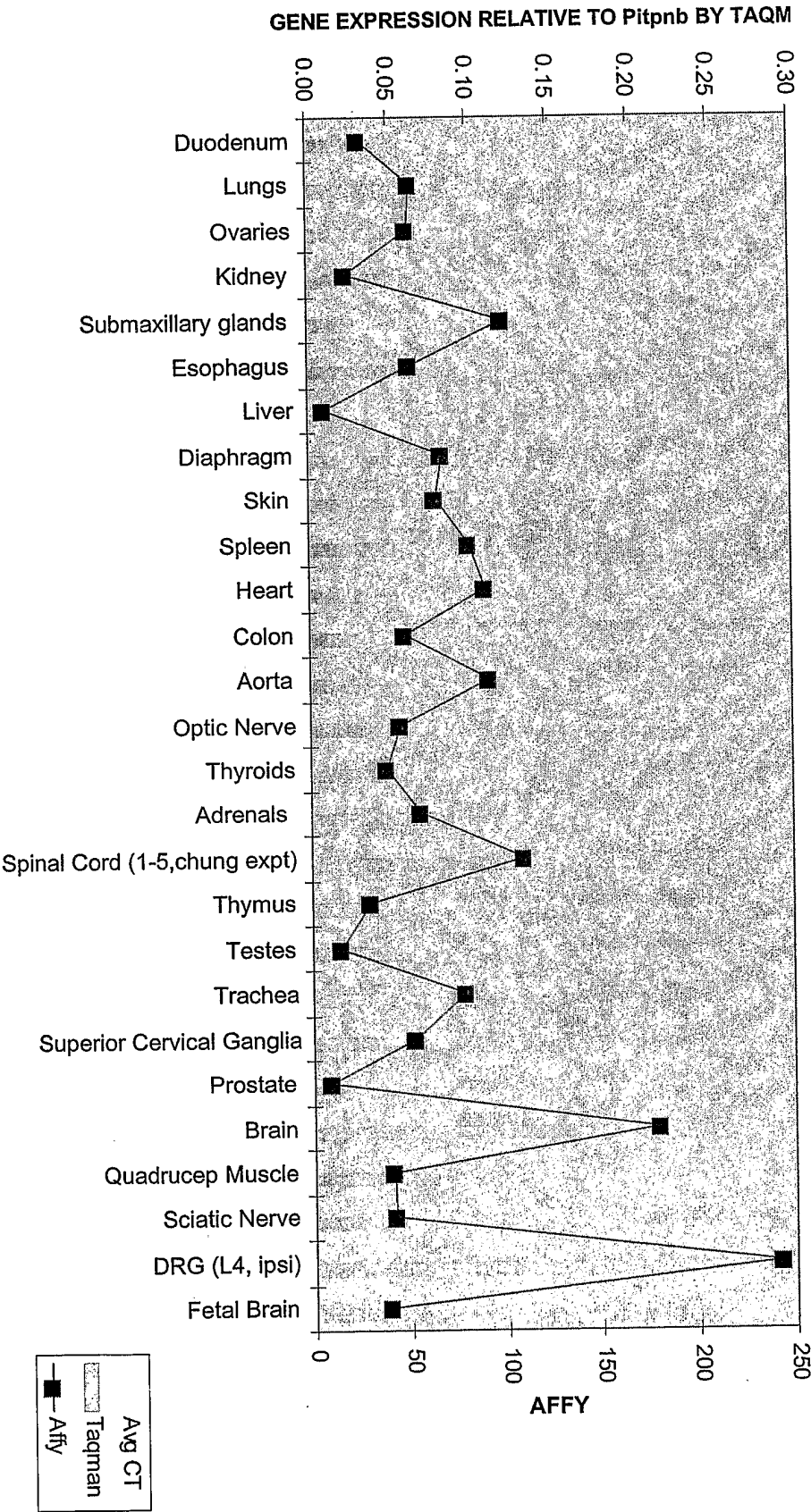
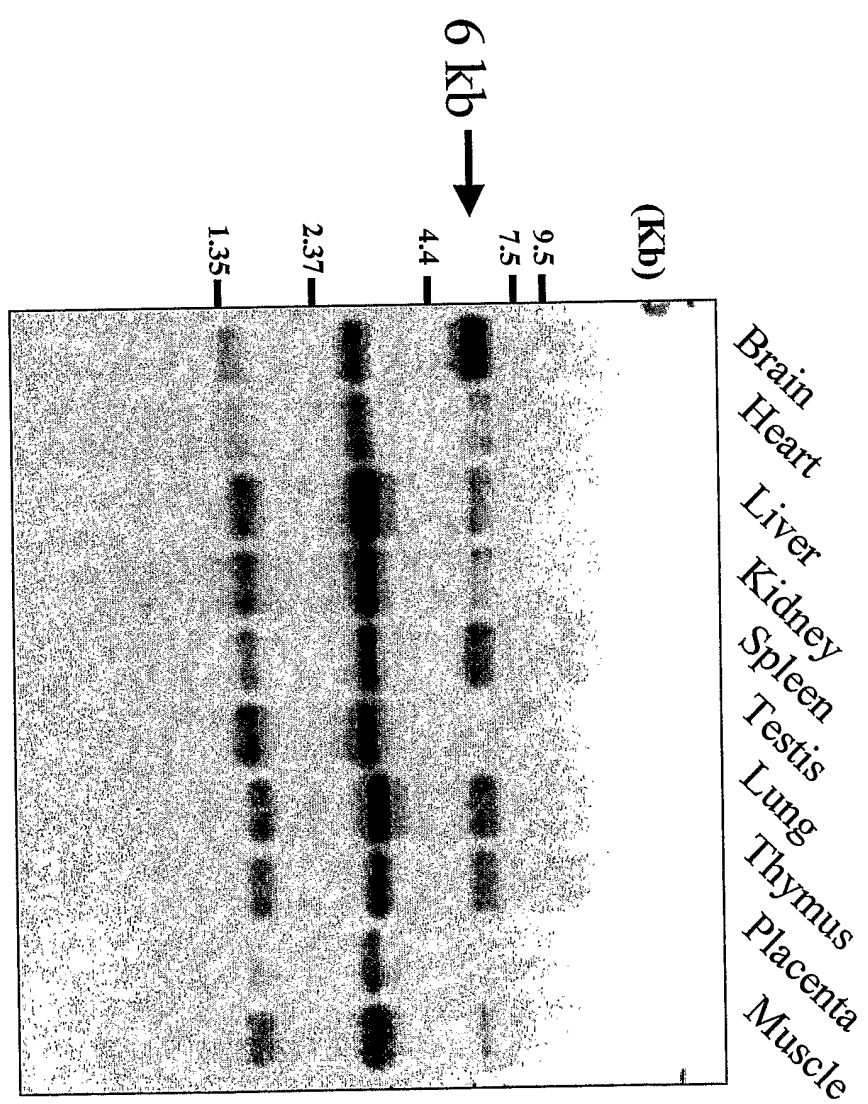


Figure 1B

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Figure 2

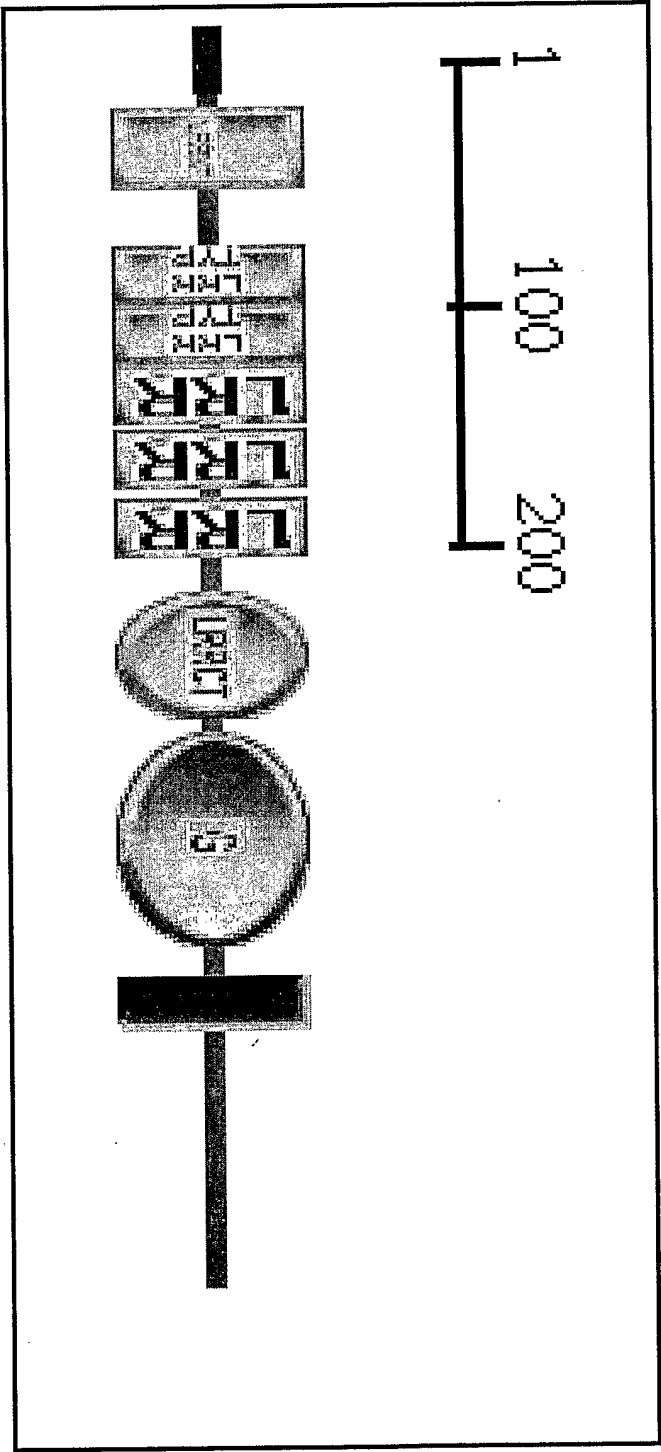


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Figure 3

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human PNPg5	(1)	MOPODRLRGIMLITLSTLLEFEVARAGRPVYGGPANGICASNILSSGSKOOLPNVPOSILBQVYALIDLSHNNTSRLRAEWTPTRLTNLHS		
human AB032989	(1)	MOPODRLRGIMLITLSTLLEFEVARAGRPVYGGPANGICASNILSSGSKOOLPNVPOSILBQVYALIDLSHNNTSRLRAEWTPTRLTNLHS		
mouse PNPg5	(1)	MOPODRLRGIMLITLSTLLEFEVARAGRPVYGGPANGICASNILSSGSKOOLPNVPOSILBQVYALIDLSHNNTSRLRAEWTPTRLTNLHS		
mouse AK035960	(1)	MOPODRLRGIMLITLSTLLEFEVARAGRPVYGGPANGICASNILSSGSKOOLPNVPOSILBQVYALIDLSHNNTSRLRAEWTPTRLTNLHS		180
PNPG5 CONTIGab	(91)	91	LLLSHNHNLNFISSRAFPVYPNIRYLDLSNNHLITLDEPLFSGTQALFVLLYNNHIVVVDNAFEDMAOLQKLYSONMTSRFPILITKD	
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human PNPg5	(91)	LLLSHNHNLNFISSRAFPVYPNIRYLDLSNNHLITLDEPLFSGTQALFVLLYNNHIVVVDNAFEDMAOLQKLYSONMTSRFPILITKD		
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mouse PNPg5	(91)	LLLSHNHNLNFISSRAFPVYPNIRYLDLSNNHLITLDEPLFSGTQALFVLLYNNHIVVVDNAFEDMAOLQKLYSONMTSRFPILITKD		
mouse AK035960	(91)	LLLSHNHNLNFISSRAFPVYPNIRYLDLSNNHLITLDEPLFSGTQALFVLLYNNHIVVVDNAFEDMAOLQKLYSONMTSRFPILITKD		270
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rat PNPg5	(181)	SSSLPRLITLIDLSNNKIKKLPIDLOKLPRAWYNGVLYHNHNPFEDECKLYOLFSHWQYROLSSVMDFOEDLYCGHSKRLHNVSILDFENG		
human PNPg5	(181)	SSSLPRLITLIDLSNNKIKKLPIDLOKLPRAWYNGVLYHNHNPFEDECKLYOLFSHWQYROLSSVMDFOEDLYCGHSKRLHNVSILDFENG		
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PNPG5 CONTIGab	(271)	271	SEYKESAWEAHIGDITLTIIDDTKQOQGMTKVWVTPSNEBOVLNQGANGTVLVSDEGNTLHEKENOYVEDGGVYTCYAMGETENETISVELKAVN	
rat PNPg5	(271)	SEYKESAWEAHIGDITLTIIDDTKQOQGMTKVWVTPSNEBOVLNQGANGTVLVSDEGNTLHEKENOYVEDGGVYTCYAMGETENETISVELKAVN		
human PNPg5	(271)	SEYKESAWEAHIGDITLTIIDDTKQOQGMTKVWVTPSNEBOVLNQGANGTVLVSDEGNTLHEKENOYVEDGGVYTCYAMGETENETISVELKAVN		
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mouse PNPg5	(271)	SEYKESAWEAHIGDITLTIIDDTKQOQGMTKVWVTPSNEBOVLNQGANGTVLVSDEGNTLHEKENOYVEDGGVYTCYAMGETENETISVELKAVN		
mouse AK035960	(271)	SEYKESAWEAHIGDITLTIIDDTKQOQGMTKVWVTPSNEBOVLNQGANGTVLVSDEGNTLHEKENOYVEDGGVYTCYAMGETENETISVELKAVN		450
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rat PNPg5	(361)	FTLHGHNDLTNTAYTTLVAGCLISVYVLLIYXLYLTPRCGWRGVEKEPSSHQGDSLSSSMTSTTPNHDPMAGEDKDDGDRVAFLLEPARGG		
human PNPg5	(361)	FTLHGHNDLTNTAYTTLVAGCLISVYVLLIYXLYLTPRCGWRGVEKEPSSHQGDSLSSSMTSTTPNHDPMAGEDKDDGDRVAFLLEPARGG		
human AB032989	(305)	FTLHGHNDLTNTAYTTLVAGCLISVYVLLIYXLYLTPRCGWRGVEKEPSSHQGDSLSSSMTSTTPNHDPMAGEDKDDGDRVAFLLEPARGG		
mouse PNPg5	(360)	FTLHGHNDLTNTAYTTLVAGCLISVYVLLIYXLYLTPRCGWRGVEKEPSSHQGDSLSSSMTSTTPNHDPMAGEDKDDGDRVAFLLEPARGG		
mouse AK035960	(360)	FTLHGHNDLTNTAYTTLVAGCLISVYVLLIYXLYLTPRCGWRGVEKEPSSHQGDSLSSSMTSTTPNHDPMAGEDKDDGDRVAFLLEPARGG		494
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human PNPg5	(451)	OGONGKLPKGNLTPVPEATGKGRMSDPESVSSVSDTPPLV-		
human AB032989	(395)	OGONGKLPKGNLTPVPEATGKGRMSDPESVSSVSDTPPLV-		
mouse PNPg5	(450)	OGONGKLPKGNLTPVPEATGKGRMSDPESVSSVSDTPPLV-		
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Figure 4



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Figure 5

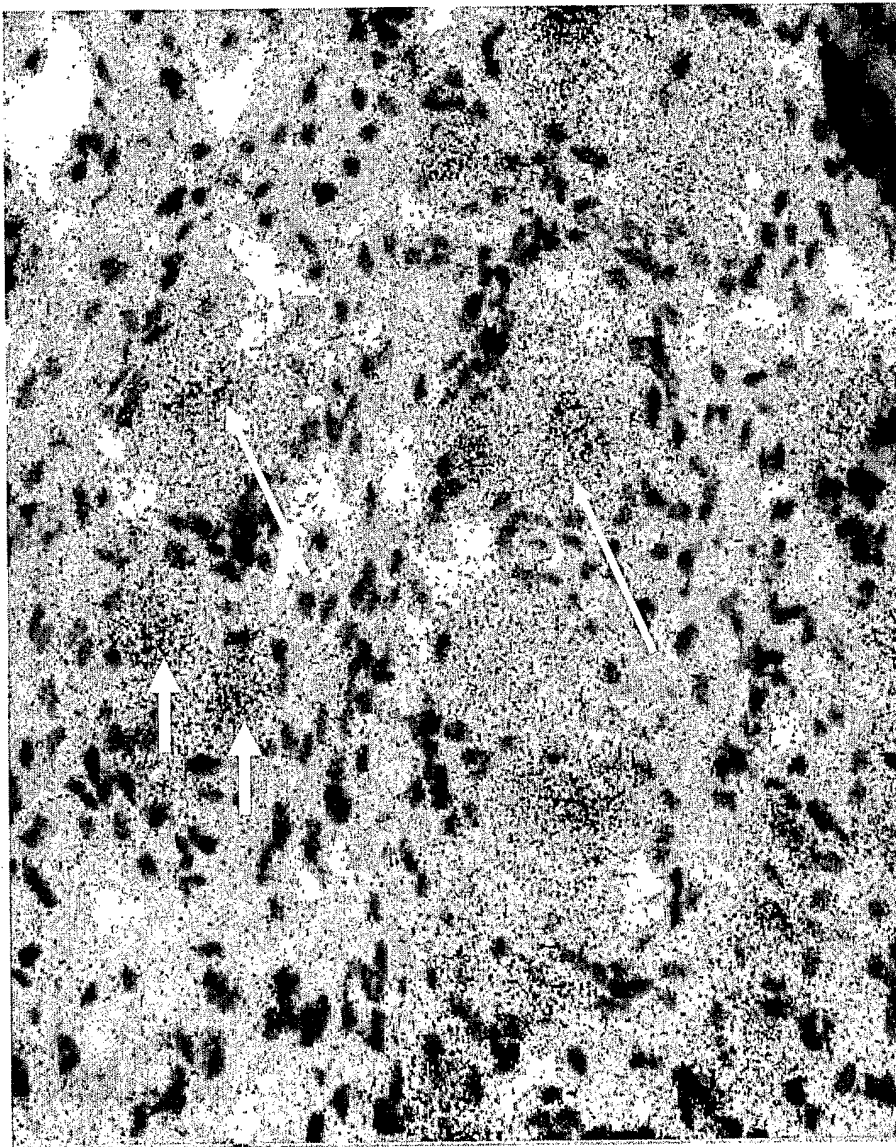
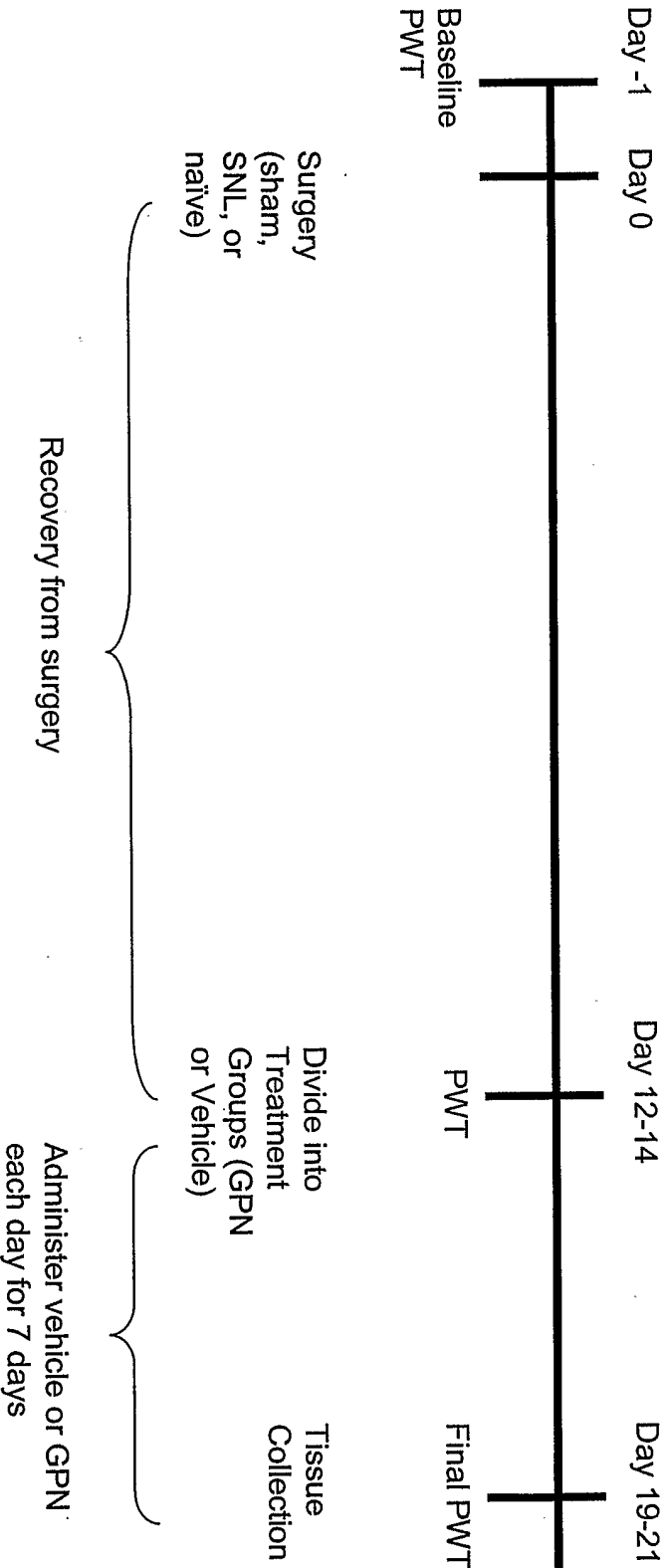
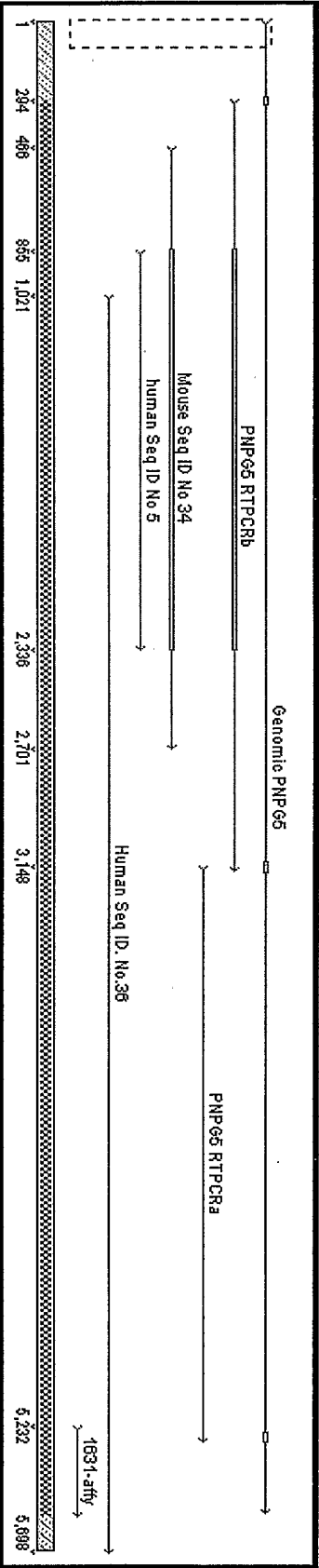


Figure 6



PWT = paw withdrawal threshold (paw pressure test)
GPN = 100 mg/kg gabapentin i.p.

Figure 7



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Figure 8A

Protein-protein interaction between PNP2 and PNP5

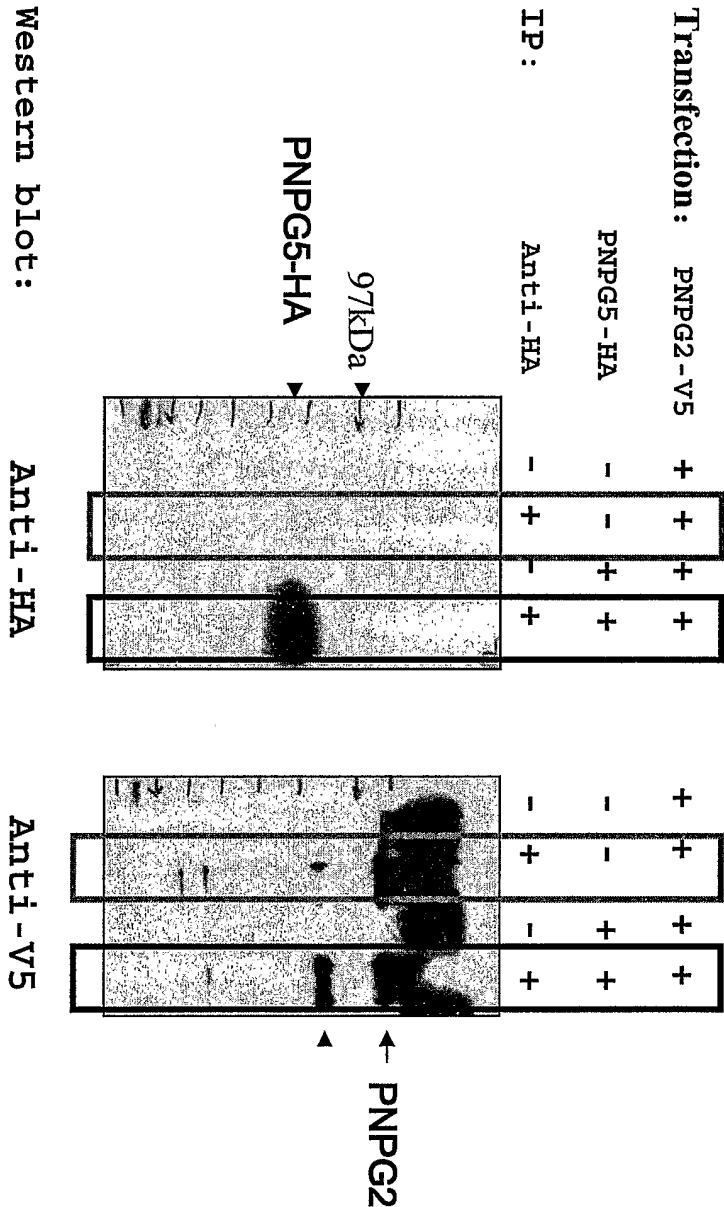


Figure 8B

Protein-protein interaction between PNP G2 and PNP G5

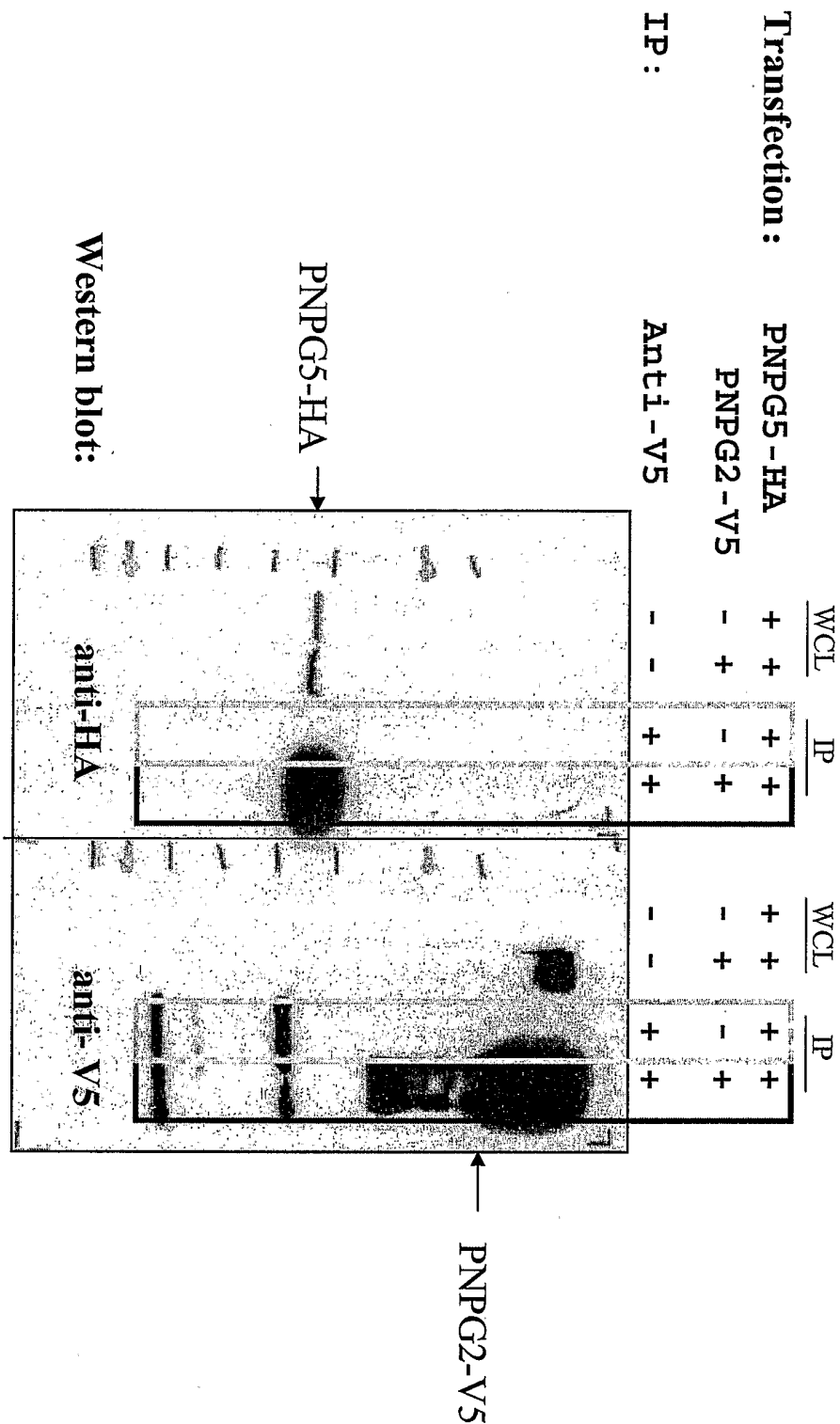
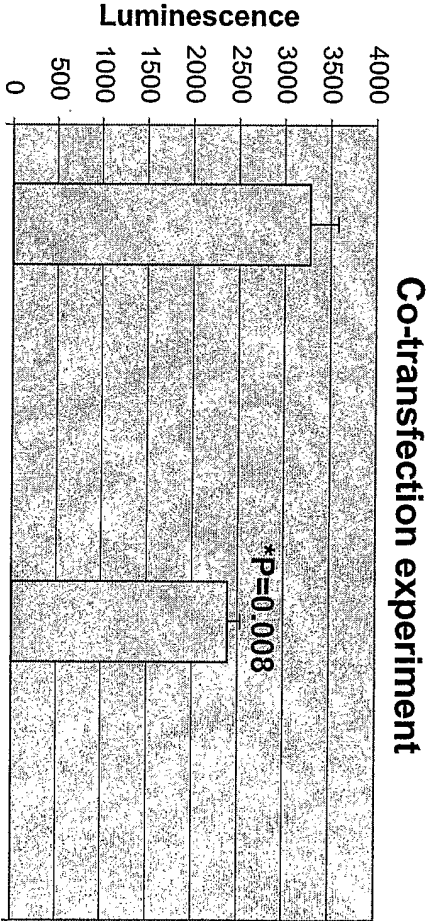
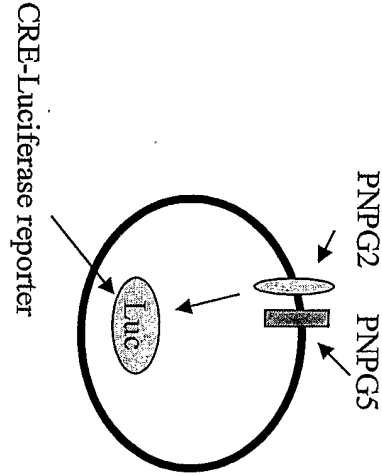


Figure 9

PNPG5: ligand/co-factor for PNPg2-mediated signaling

A. Intracellular-activation



B. Intercellular activation

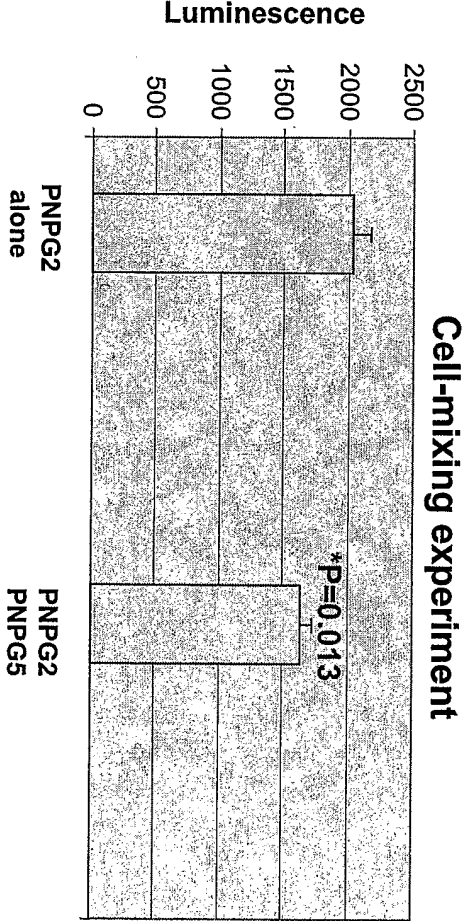
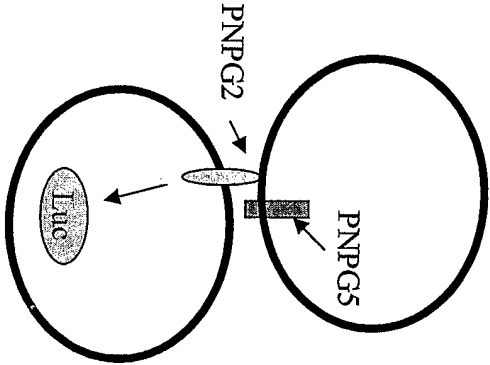
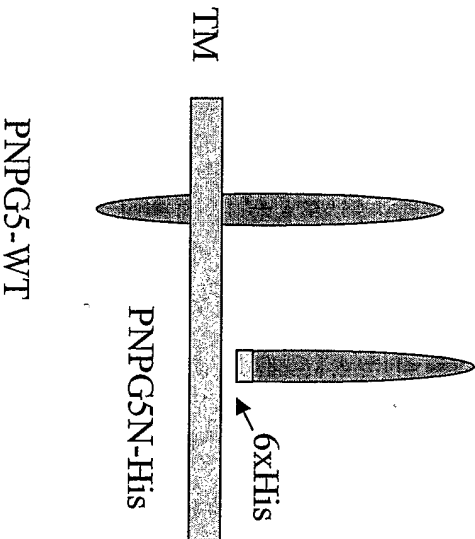


Figure 10

Soluble PNP G5N-His



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Tong, Jiefei

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Lys Gln Gln Leu Pro Asn Val Pro Gln Ser Leu Pro Ser Tyr Thr Ala
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Leu Leu Asp Leu Ser His Asn Asn Leu Ser Arg Leu Arg Ala Glu Trp
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Thr Pro Thr Arg Leu Thr Asn Leu His Ser Leu Leu Leu Ser His Asn
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His Leu Asn Phe Ile Ser Ser Glu Ala Phe Val Pro Val Pro Asn Leu
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Arg Tyr Leu Asp Leu Ser Ser Asn His Leu His Thr Leu Asp Glu Phe
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Leu Phe Ser Asp Leu Gln Ala Leu Glu Val Leu Leu Leu Tyr Asn Asn
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His Ile Val Val Val Asp Arg Asn Ala Phe Glu Asp Met Ala Gln Leu
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Gln Lys Leu Tyr Leu Ser Gln Asn Gln Ile Ser Arg Phe Pro Val Glu
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 Pro Ala Trp Val Lys Asn Gly Leu Tyr Leu His Asn Asn Pro Leu Glu
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 Cys Asp Cys Lys Leu Tyr Gln Leu Phe Ser His Trp Gln Tyr Arg Gln
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 245 250 255
 Lys Lys Leu His Asn Ile Phe Ser Leu Asp Phe Phe Asn Cys Ser Glu
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 Tyr Lys Glu Ser Ala Trp Glu Ala His Leu Gly Asp Thr Leu Thr Ile
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 Arg Asn Gly Asp Leu Phe Phe Lys Lys Val Gln Val Glu Asp Gly Gly
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 Val Tyr Thr Cys Tyr Ala Met Gly Glu Thr Phe Asn Glu Thr Leu Ser
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 Val Glu Leu Lys Val Tyr Asn Phe Thr Leu His Gly His His Asp Thr
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 Gly Val Glu Lys Pro Ser Ser His Gln Gly Asp Ser Leu Ser Ser Ser
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 Met Leu Ser Thr Thr Pro Asn His Asp Pro Met Ala Gly Gly Asp Lys
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Gly Gln Gly Gln Asn Gly Lys Leu Lys Pro Gly Asn Thr Leu Pro Val
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Leu Leu Asp Leu Ser His Asn Asn Leu Ser Arg Leu Arg Ala Glu Trp
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Thr Pro Thr Arg Leu Thr Gln Leu His Ser Leu Leu Leu Ser His Asn
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His Leu Asn Phe Ile Ser Ser Glu Ala Phe Ser Pro Val Pro Asn Leu
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Arg-Tyr-Leu-Asp-Leu-Ser-Ser-Asn-Gln-Leu-Arg-Thr-Leu-Asp-Glu-Phe
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Gln Lys Leu Tyr Leu Ser Gln Asn Gln Ile Ser Arg Phe Pro Leu Glu
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Ser Ser Asn Lys Leu Lys Asn Leu Pro Leu Pro Asp Leu Gln Lys Leu
 195 200 205

Pro Ala Trp Ile Lys Asn Gly Leu Tyr Leu His Asn Asn Pro Leu Asn
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Cys Asp Cys Glu Leu Tyr Gln Leu Phe Ser His Trp Gln Tyr Arg Gln
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Leu Ser Ser Val Met Asp Phe Gln Glu Asp Leu Tyr Cys Met Asn Ser
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Lys Lys Leu His Asn Val Phe Asn Leu Ser Phe Leu Asn Cys Gly Glu
 260 265 270

Tyr Lys Glu Arg Ala Trp Glu Ala His Leu Gly Asp Thr Leu Ile Ile
 275 280 285

Lys Cys Asp Thr Lys Gln Gln Gly Met Thr Lys Val Trp Val Thr Pro
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Ser Asn Glu Arg Val Leu Asp Glu Val Thr Asn Gly Thr Val Ser Val
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Ser Lys Asp Gly Ser Leu Leu Phe Gln Gln Val Gln Val Glu Asp Gly
 325 330 335

Gly Val Tyr Thr Cys Tyr Ala Met Gly Glu Thr Phe Asn Glu Thr Leu
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Ser Val Glu Leu Lys Val His Asn Phe Thr Leu His Gly His His Asp
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Val Leu Val Leu Ile Tyr Leu Tyr Leu Thr Pro Cys Arg Cys Trp Cys
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Arg Gly Val Glu Lys Pro Ser Ser His Gln Gly Asp Ser Leu Ser Ser
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Ser Met Leu Ser Thr Thr Pro Asn His Asp Pro Met Ala Gly Gly Asp
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 Phe Ser His Trp Gln Tyr Arg Gln Leu Ser Ser Val Met Asp Phe Gln
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 Glu Asp Leu Tyr Cys Met Asn Ser Lys Lys Leu His Asn Val Phe Asn
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 Leu Pro Ala Ser Leu Leu His Arg Leu Arg Phe Leu Glu Glu Leu Arg
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 Leu Ala Gly Asn Ala Leu Thr His Ile Pro Lys Gly Ala Phe Ala Gly
 100 105 110
 Leu His Ser Leu Lys Val Leu Met Leu Gln Asn Asn Gln Leu Arg Gln
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 Val Pro Glu Glu Ala Leu Gln Asn Leu Arg Ser Leu Gln Ser Leu Arg
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 Leu Asp Ala Asn His Ile Ser Tyr Val Pro Pro Ser Cys Phe Ser Gly
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 Leu His Ser Leu Arg His Leu Trp Leu Asp Asp Asn Ala Leu Thr Asp
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 Val Pro Val Gln Ala Phe Arg Ser Leu Ser Ala Leu Gln Ala Met Thr
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 Leu Ser Ser Leu Val Val Leu His Leu His Asn Asn Arg Ile His Ser
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 Pro Glu Arg Ala Phe Val Gly Asn Pro Ser Leu Ile Thr Ile His Phe
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Phe Pro Asp Leu Thr Gly Thr Ala Thr Leu Glu Ser Leu Thr Leu Thr
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Asn Leu Gln Val Leu Asp Leu Ser Tyr Asn Leu Leu Glu Asp Leu Pro
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Ser Leu Ser Gly Cys Gln Lys Leu Gln Lys Ile Asp Leu Arg His Asn
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Glu Ile Tyr Glu Ile Lys Gly Gly Thr Phe Gln Gln Leu Phe Asn Leu
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Arg Ser Leu Asn Leu Ala Arg Asn Lys Ile Ala Ile Ile His Pro Asn
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Ala Phe Ser Thr Leu Pro Ser Leu Ile Lys Leu Asp Leu Ser Ser Asn
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Leu Leu Ser Ser Phe Pro Val Thr Gly Leu His Gly Leu Thr His Leu
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 675 680 685
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 690 695 700
 Glu Tyr Asn Ala Ser Pro Leu Cys Leu Pro Leu Pro Phe Gly Glu Pro
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 Ala Leu Leu Leu Phe Thr Asn Cys Ile Leu Tyr Cys Pro Val Ala Phe
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 Leu Ser Phe Ser Ser Leu Leu Asn Leu Thr Phe Ile Ser Pro Glu Val
 Page 48

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Ser Leu Gly Lys Gln Thr Arg Phe Trp Thr Arg Ala Lys His Pro Ser
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Thr Gln Ala Leu Val Ser Phe Thr His Ala Ser Ile Ala Tyr Asp Leu
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Thr Pro Thr Arg Leu Thr Asn Leu His Ser Leu Leu Leu Ser His Asn
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His Leu Asn Phe Ile Ser Ser Glu Ala Phe Val Pro Val Pro Asn Leu
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Arg Tyr Leu Asp Leu Ser Ser Asn His Leu His Thr Leu Asp Glu Phe
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115

120

125

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Gln Lys Leu Tyr Leu Ser Gln Asn Met Ile Ser Arg Phe Pro Leu Glu
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Leu Ile Lys Asp Ala Ser Arg Leu Pro Lys Leu Thr Leu Leu Asp Leu
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Ser Ser Asn Lys Leu Lys Lys Leu Pro Leu Thr Asp Leu Gln Lys Leu
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Cys Asp Cys Lys Leu Tyr Gln Leu Phe Ser His Trp Gln Tyr Arg Gln
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Ser Val Glu Leu Lys Val Tyr Asn Phe Thr Leu His Gly His His Asp
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 405 410 415

Ser Met Leu Ser Thr Thr Pro Asn His Asp Pro Met Ala Gly Gly Asp
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Lys Asp Asp Gly Phe Asp Arg Arg Val Ala Phe Leu Glu Pro Ala Gly
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