Title: METHODS FOR THE TREATMENT OF CHRONIC PAIN AND COMPOSITIONS THEREFOR

Abstract: The invention discloses ESDN as a suitable target for the development of new therapeutics to treat or ameliorate chronic pain. The invention relates to methods to treat and/or ameliorate chronic pain and pharmaceutical compositions therefor comprising modulators with inhibitory effect on ESDN activity and/or ESDN gene expression. The invention also relates to a method to identify compounds with therapeutic usefulness to treat chronic pain, comprising identifying compounds that can inhibit ESDN activity and/or gene expression which can also reverse the pathological effects of chronic pain in vivo.
METHODS FOR THE TREATMENT OF CHRONIC PAIN
AND COMPOSITIONS THEREFOR

BACKGROUND OF THE INVENTION

Pain is a term that encompasses a spectrum of clinical states. Under normal
conditions acute pain is beneficial and serves as a physiological warning for a potentially
tissue-damaging situation. More persistent pain, usually associated with inflammation,
can also be regarded as a normal protective response to mild tissue injury and resolves
when the injury has healed. However, chronic pain occurs when the stimulus and pain
are unrelated and the pain is no longer a protective mechanism. These types of pain
syndromes (e.g. rheumatoid arthritis, cancer pain, neuropathic pain) are notoriously
difficult to treat. It is estimated that 10-20% of the adult population suffers from chronic
pain. To date, the main analgesics employed are based on opiates and non-steroidal anti-
inflammatory drugs (NSAIDS) such as aspirin. Both classes of drugs can produce severe
side-effects; NSAIDS can cause gastric ulceration and renal damage while opiates can
cause nausea, constipation, confusion and dependency problems. Despite these
disadvantages, no new class of analgesics have been discovered or developed recently;
there is clearly a need for additional therapies for chronic pain.

Chronic pain states are characterised by a number of clinical features. As well as
spontaneous pain, patients may exhibit hyperalgesia (a greatly exaggerated response to a
noxious mechanical, hot, or cold stimulus), and allodynia (previously non-noxious stimuli
are now perceived as painful). All these features result from a complex series of events
involving changes in the function of sensory nerves in the periphery and in the processing
of sensory information in the spinal cord and brain. These changes occur in response to
direct neuronal damage or in response to mediators released during tissue damage or
inflammation.

Broadly speaking, chronic pain syndromes can be defined as inflammatory (also
known as nociceptive) or neuropathic. Chronic inflammatory pain, as its name suggests,
ocurs during conditions in which there is underlying inflammation such as rheumatoid
arthritis, burns, muscle damage or surgical wounds. Knowledge of the mechanisms
underlying inflammatory pain has advanced considerably over recent years and it is known to involve a variety of mediators and their activation and sensitization of the peripheral terminals of sensory nerves and the consequent longer term changes in reactivity of spinal cord neurons.

Chronic neuropathic pain is caused where there is a primary lesion or dysfunction of the nervous system and occurs, for example, during conditions such as trigeminal neuralgia, diabetic neuropathy, post-herpetic neuralgia, amputation or physical nerve damage. Chronic neuropathic pain results from damage to nerves by trauma, by diseases such as diabetes, herpes zoster, or late-stage cancer (see below), or by chemical injury (e.g. some anti-HIV drugs). It may also develop after amputation (including mastectomy), and is involved in some low-back pain. The mechanisms of chronic neuropathic pain are poorly understood but are thought to involve spontaneous firing of sensory nerves due to the novel expression of certain classes of ion channel, sprouting of sensory fibres into different layers of the spinal cord, and changes in the expression of various neurotransmitters and receptors in the sensory nerves and spinal cord. Traditionally chronic neuropathic pain has proven to be intractable and is resistant to the standard non-steroidal and opiate analgesics. There is therefore clearly an unmet clinical need for new analgesics to treat this type of pain.

Cancer pain is the most common chronic pain syndrome (with probably inflammatory and neuropathic components). It is estimated that one third of patients with advanced cancer will develop skeletal metastases, particularly in breast, prostate and lung cancer. Metastatic bone disease commonly results in bone pain that is usually located to a discrete area and is described as a deep, boring sensation that aches and burns, accompanied by episodes of stabbing discomfort. The mechanisms responsible for bone cancer pain are unknown but it probably involves structural damage, periosteal irritation and nerve entrapment. There is evidence for the disruption of normal bone metabolism and the production of inflammatory prostaglandins and cytokines. Current treatment of bone cancer pain rests with opiates but the doses required results in unacceptable side-effects and at least 20 % of patients still have uncontrolled pain. Novel, well tolerated and effective analgesics are desired to optimise the quality of life of these patients (Coleman RE (1997) Cancer 80; 1588-1594).
Osteoarthritis pain is the most common form of chronic neuropathic pain (with probably inflammatory and neuropathic components) for which people visit general practitioners. Osteoarthritis is a chronic disease involving progressive structural changes in joint tissues, principally cartilage, synovium and subchondral bone. Typically, arthritic joints exhibit cartilage oedema and erosion, subchondral bone and synovial thickening, and formation of bony osteophytes, all contributing to deformation of the articular surface. The principal clinical symptom of osteoarthritis is pain, although the mechanisms underlying the chronic neuropathic pain in this condition are not understood.

Traditionally, attempts have been made to alleviate chronic neuropathic pain by directing therapeutic compounds to sensory fibers involved in pain signaling, e.g., the "C fiber", (Wooll C.J. et al. (1995) J. Comp. Neurol. 360, 121-124.) or to the sensory fibers that transmit noxious information along the spinal cord (Dickenson AH. & Sullivan A. (1987) *Neuropharmacol.* 26; 1235-1238.). It has also been postulated that compounds may alleviate this pain by blocking mediator release (e.g. cytokines and bradykinins) from tissue during inflammation and/or blocking the receptors for these mediators (Dray A. & Urban L. (1996) *Annu. Rev. Pharmacol. Toxicol.* 36; 253-280.).

We have now surprisingly discovered that mRNA for the recently discovered membrane protein ESDN, (endothelial and smooth muscle cell-derived neuropilin-like molecule; see Kobuke et al,(2001) J Biol. Chem. 276: 34105-34114), is up regulated in animal models of chronic pain. Thus, ESDN can be used as a novel drug target for chronic pain. The invention also provides a method for identifying modulators that inhibit ESDN activity and/or inhibit ESDN gene expression and the use of such modulators for the treatment of chronic pain in human and veterinary patients. The invention also provides pharmaceutical compositions comprising said modulators.

**SUMMARY OF THE INVENTION**

The instant application relates to the discovery that ESDN is a suitable target for the development of new therapeutics to treat or ameliorate chronic pain. Thus, in one aspect the invention relates to a method to identify modulators useful to treat or ameliorate chronic pain, including chronic neuropathic pain, comprising: a) assaying for the ability of a candidate
modulator to inhibit the activity of ESDN and/or inhibit ESDN gene expression in vitro or in vivo and which can further include b) assaying for the ability of an identified inhibitory modulator to reverse the pathological effects observed in animal models of chronic pain and/or in clinical studies with subjects with chronic pain.

In another aspect, the invention relates to a method to treat or ameliorate chronic pain, including chronic neuropathic pain, comprising administering to a subject in need thereof an effective amount of a ESDN modulator, wherein said modulator, e.g., inhibits the activity of ESDN and/or inhibits ESDN gene expression in said subject. In one embodiment the modulator comprises any one or more substances selected from the group consisting of antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamers, siRNA and double or single stranded RNA wherein said substances are designed to inhibit ESDN gene expression. In a further embodiment, the modulator comprises antibodies to ESDN or fragments thereof, wherein said antibodies can e.g., inhibit ESDN activity.

In another aspect, the invention relates to a method to treat or ameliorate chronic pain, including chronic neuropathic pain, comprising administering to a subject in need thereof a pharmaceutical composition comprising an effective amount of a ESDN modulator. In various embodiments, said pharmaceutical composition comprises any of the ESDN modulators discussed above.

In another aspect, the invention relates to a pharmaceutical composition comprising a ESDN modulator in an amount effective to treat or ameliorate chronic pain, including chronic neuropathic pain, in a subject in need thereof wherein said modulator, e.g., can inhibit the activity of ESDN and/or inhibit ESDN gene expression. In one embodiment, said pharmaceutical composition comprises any one or more substances selected from the group consisting of antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamers, siRNA or double or single stranded RNA directed to a nucleic acid sequence of ESDN wherein said substances are designed to inhibit ESDN gene expression. In a further embodiment, said pharmaceutical composition comprises antibodies to ESDN or fragments thereof, wherein said antibodies can, e.g., inhibit ESDN activity.
In another aspect, the invention relates to a method to diagnose subjects suffering from chronic pain who may be suitable candidates for treatment with ESDN modulators comprising detecting levels of this protein in a biological sample from said subject wherein subjects with increased levels compared to controls would be suitable candidates for ESDN modulator treatment.

In yet another aspect, the invention relates to a method to diagnose subjects suffering from chronic pain who may be suitable candidates for treatment with ESDN modulators comprising assaying mRNA levels of this protein in a biological sample from said subject wherein subjects with increased levels compared to controls would be suitable candidates for ESDN modulator treatment.

In yet another aspect, there is provided a method to treat or ameliorate chronic pain, including chronic neuropathic pain, comprising: (a) assaying for ESDN mRNA and/or protein levels in a subject; and (b) administering to a subject with increased levels of ESDN mRNA and/or protein levels compared to controls a ESDN modulator in an amount sufficient to treat or ameliorate the pathological effects of chronic pain.

In yet another aspect of the present invention there are provided assay methods and kits comprising the components necessary to detect expression of polynucleotides encoding ESDN or related regulatory polypeptides, or levels of ESDN or related regulatory polypeptides, or fragments thereof, in body tissue samples derived from a patient, such kits comprising, e.g., antibodies that bind to said polypeptides, or to fragments thereof, or oligonucleotide probes that hybridize with said polynucleotides. In a preferred embodiment, such kits also comprise instructions detailing the procedures by which the kit components are to be used.

The present invention also pertains to the use of a ESDN modulator in the manufacture of a medicament for the treatment or amelioration of chronic pain, including chronic neuropathic pain. In one embodiment, said ESDN modulator comprises any one or more substances selected from the group consisting of antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamer, si RNA and double or single stranded RNA wherein said substances are designed to inhibit ESDN gene expression. In yet a further embodiment,
said ESDN modulator comprises one or more antibodies to ESDN, or fragments thereof, wherein said antibodies or fragments thereof can, e.g., inhibit ESDN activity.

The invention also pertains to a ESDN modulator for use as a pharmaceutical. In one embodiment, said ESDN modulator comprises any one or more substances selected from the group consisting of antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamer, siRNA and double or single stranded RNA wherein said substances are designed to inhibit ESDN gene expression. In yet a further embodiment, said ESDN modulator comprises one or more antibodies to ESDN, or fragments thereof, wherein said antibodies or fragments thereof can, e.g., inhibit ESDN activity.

DESCRIPTION OF THE FIGURES

Figure 1. Band 31 sequence contig composed of 11 sequences from bands 31 and 28. The regions used for designing genes-specific primers for RT-PCR confirmation are in bold-face and underlined.

Figure 2. Cloning of band 31 cDNA by RACE-PCR. A. Schematic presentation of the products from three rounds of RACE PCR experiments to generate a 2450 bp contig sequence of band 31. Relative position of each end of the PCR products in the contig sequence are indicated at the bottom of the diagram B. Nucleotide sequence of the 2450 bp contig. The regions for three rounds of RACE PCR primer design are underlined and in boldface. The LINE repeat region is marked in bold italics.

DETAILED DESCRIPTION OF THE INVENTION

It is contemplated that the invention described herein is not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention in any way.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this
invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices and materials are now described. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing the materials and methodologies that are reported in the publication which might be used in connection with the invention.


As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to the "antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

"Pathological effects of chronic pain" include, but are not limited to, hyperalgesia and allodynia.

The ability of a substance to "modulate" ESDN (e.g., a ESDN modulator) includes, but is not limited to, the ability of a substance to inhibit the activity of ESDN and/or inhibit ESDN gene expression. Such modulation could also involve effecting the ability of other proteins to
interact with ESDN for example related regulatory proteins or proteins that are modified by ESDN.

"Nucleic acid sequence", as used herein, refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin that may be single or double stranded, and represent the sense or antisense strand.

The term "antisense" as used herein, refers to nucleotide sequences which are complementary to a specific DNA or RNA sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense' strand. Antisense molecules may be produced by any method, including synthesis by ligating the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a complementary strand. Once introduced into a cell, this transcribed strand combines natural sequences produced by the cell to form duplexes. These duplexes then block either the further transcription or translation. The designation "negative " is sometimes used in reference to the antisense strand, and "positive" is sometimes used in reference to the sense strand.

As contemplated herein, antisense oligonucleotides, triple helix DNA, RNA aptamers, ribozymes, siRNA and double or single stranded RNA are "directed to a nucleic acid sequence of ESDN" such that the nucleotide sequence of ESDN chosen will produce gene-specific inhibition of ESDN gene expression. For example, knowledge of the ESDN nucleotide sequence may be used to design an antisense molecule which gives strongest hybridization to the mRNA. Similarly, ribozymes can be synthesized to recognize specific nucleotide sequences of ESDN and cleave it (Cech. J. Amer. Med Assn. 260:3030 (1988). Techniques for the design of such molecules for use in targeted inhibition of gene expression is well known to one of skill in the art.

The term "ESDN" refers to any and all forms of this polypeptide including, but not limited to, partial forms, isoforms, precursor forms, the full length polypeptide, fusion proteins containing the ESDN sequence or fragments of any of the above, from human or any other species. The sequence of ESDN may be found in Genbank, Accession Numbers AF387547
(human), AF387548 (mouse), AF387549 (rat). Homologs and orthologs of ESDN, which would be apparent to one of skill in the art, are meant to be included in this definition. It is also contemplated that the term refers to ESDN isolated from naturally occurring sources of any species such as genomic DNA libraries as well as genetically engineered host cells comprising expression systems, or produced by chemical synthesis using, for instance, automated peptide synthesizers or a combination of such methods. Means for isolating and preparing such polypeptides are well understood in the art.

The term "sample" as used herein, is used in its broadest sense. A biological sample from a subject may comprise blood, urine or other biological material with which ESDN activity or gene expression may be assayed. A biological sample may include dorsal root ganglia from which total RNA may be purified for gene expression profiling using conventional glass chip microarray technologies such as Affymetrix chips, RT-PCR or other conventional methods.

As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fα, F(ab')2, and Fv, which are capable of binding the epitopic determinant. Antibodies that bind ESDN polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptides or peptides used to immunize an animal can be derived from the translation of RNA or synthesized chemically, and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin. The coupled peptide is then used to immunize an animal (e.g., a mouse, goat, chicken, a rat or a rabbit).

The term "humanized antibody" as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody, while still retaining the original binding ability.

A "therapeutically effective amount" is the amount of drug sufficient to treat and/or ameliorate the pathological effects of chronic pain, including but not limited to, hyperalgesia.
"Related regulatory proteins" and "related regulatory polypeptides" as used herein refer to polypeptides involved in the regulation of ESDN which may be identified by one of skill in the art using conventional methods such as described herein.

Pain as defined herein includes chronic pain. "Chronic pain" includes inflammatory (nociceptive) and neuropathic pain as described above.

"Subject" refers to any human or nonhuman organism.

The invention is based on the surprising discovery that ESDN messenger RNA is up regulated in rat models of chronic neuropathic pain. Thus, ESDN is a useful drug target for the development of therapeutics for the treatment of chronic pain, a disease state not previously known to involve ESDN.

Thus, in one aspect the invention relates to a method to identify modulators useful to treat or ameliorate chronic pain, including chronic neuropathic pain, comprising: a) assaying for the ability of a candidate modulator to inhibit the activity of ESDN and/or inhibit ESDN gene expression in vitro or in vivo and which can further include b) assaying for the ability of an identified inhibitory modulator to reverse the pathological effects observed in animal models of chronic pain and/or in clinical studies with subjects with chronic pain.

Conventional screening assays (both in vitro and in vivo) may be used to identify modulators that inhibit ESDN activity and/or inhibit ESDN gene expression. ESDN levels can be assayed in a subject using a biological sample from the subject using conventional protein activity assay methods. ESDN gene expression (e.g. mRNA levels) may also be determined using methods familiar to one of skill in the art, including, for example, conventional Northern analysis or commercially available microarrays. Additionally, the effect of test compound inhibition of ESDN and/or related regulatory protein levels can be detected with an ELISA antibody-based assay or fluorescent labelling reaction assay. These techniques are readily available for high throughput screening and are familiar to one skilled in the art.
Data gathered from these studies would be used to identify those modulators with therapeutic usefulness for the treatment of chronic pain as inhibitory substances could then be further assayed in conventional live animal models of chronic pain as described herein and/or in clinical trials with humans with chronic pain according to conventional methods to assess the ability of said compounds to ameliorate the pathological effects of chronic pain in vivo.

Candidate modulators for analysis according to the methods disclosed herein include chemical compounds known to possess ESDN inhibitory activity as well as compounds whose effects on this protein at any level have yet to be characterized. Compounds known to possess ESDN inhibitory activity could be directly assayed in the animal pain models described herein or in clinical trials.

In another aspect, the invention relates to a method to treat or ameliorate chronic pain comprising administering to a subject in need thereof a pharmaceutical composition comprising an effective amount of an ESDN modulator. Such modulators include antibodies directed to the ESDN polypeptide or fragments thereof. In certain particularly preferred embodiments, the pharmaceutical composition comprises antibodies that are highly selective for human ESDN polypeptides or portions of human ESDN polypeptides. Antibodies to ESDN may cause the aggregation of the protein in a subject and thus inhibit or reduce the activity of the protein. Such antibodies may also inhibit or decrease ESDN activity, for example, by interacting directly with active sites or by blocking access of substrates to active sites. ESDN antibodies may also be used to inhibit ESDN activity by preventing protein-protein interactions that may be involved in the regulation of ESDN and necessary for activity. Antibodies with inhibitory activity such as described herein can be produced and identified according to standard assays familiar to one of skill in the art.

ESDN antibodies may also be used diagnostically. For example, one could use these antibodies according to conventional methods to quantitate levels of ESDN in a subject; increased levels would indicate chronic pain and the degree of severity of this condition. Thus, different ESDN levels would be indicative of various clinical forms or severity of chronic pain. Such information would also be useful to identify subsets of patients experiencing pain that may or may not respond to treatment with ESDN inhibitors. Similarly, it is contemplated herein that quantitating the message level of ESDN in a subject
would be useful for diagnosis and determining appropriate pain therapy; subjects with increased mRNA levels of this protein compared to appropriate control individuals would be considered suitable candidates for treatment with ESDN inhibitors.

Thus in another aspect, the present invention relates to a diagnostic kit which comprises:
(a) a polynucleotide of ESDN or a fragment thereof;
(b) a nucleotide sequence complementary to that of (a);
(c) an RNAi sequence complementary to that of (a);
(d) a ESDN polypeptide, or a fragment thereof; or
(e) an antibody to a ESDN polypeptide.
It will be appreciated that in any such kit, (a), (b), (c), (d) or (e) may comprise a substantial component. It is also contemplated that said kit could comprise components (a)-(e) designed to detect levels of ESDN related regulatory proteins or proteins modified by ESDN as discussed herein.

Similarly, it is contemplated herein that monitoring ESDN protein levels or activity and/or detecting ESDN gene expression (mRNA levels) may be used as part of a clinical testing procedure, for example, to determine the efficacy of a given pain treatment regimen. For example, patients to whom pain medicine has been administered would be evaluated and the clinician would be able to identify those patients in whom ESDN levels, activity and/or gene expression levels are higher than desired (i.e. levels greater than levels in control patients not experiencing pain or in patients in whom pain has been sufficiently alleviated by clinical intervention). Based on these data, the clinician could then adjust the dosage, administration regimen or type of pain medicine prescribed. While the clinician can get an idea of the effectiveness of a particular pain medication by asking the patient how much pain he or she is experiencing, it is contemplated herein that monitoring patient levels of ESDN as described above would provide a quantitative assessment of a patient's pain level. In addition, monitoring the level of ESDN in a subject in such a way could be used to assess the level of pain experienced by nonresponsive patients (e.g. infants, comatose, burn patients). Such data could then be used by the clinician for determining the appropriate dosage, administration regimen or type of pain medication for such patients.
Factors for consideration for optimizing a therapy for a patient include the particular condition being treated, the particular mammal being treated, the clinical condition of the individual patient, the site of delivery of the active compound, the particular type of the active compound, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The therapeutically effective amount of an active compound to be administered will be governed by such considerations, and is the minimum amount necessary for the treatment of chronic pain, preferably, chronic neuropathic pain.

Suitable antibodies to ESDN or related regulatory proteins can be obtained from a commercial source or produced according to conventional methods. For example, described herein are methods for the production of antibodies capable of specifically recognizing one or more differentially expressed gene epitopes. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(\(ab\)'\)2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

For the production of antibodies to the ESDN polypeptides discussed herein, various host animals may be immunized by injection with the polypeptides, or a portion thereof. Such host animals may include, but are not limited to, rabbits, mice, goats, chickens and rats. Various adjuvants may be used to increase the immunological response, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as target gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with the polypeptides, or a portion thereof, supplemented with adjuvants as also described above.
Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD. IgY and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable or hypervariable region derived from a murine mAb and a human immunoglobulin constant region.


Most preferably, techniques useful for the production of "humanized antibodies" can be adapted to produce antibodies to the polypeptides, fragments, derivatives, and functional equivalents disclosed herein. Such techniques are disclosed in U.S. Patent Nos. 5,932,448; 5,693,762; 5,693,761; 5,585,089; 5,530,101; 5,910,771; 5,569,825; 5,625,126; 5,633,425;
5,789,650; 5,545,580; 5,661,016; and 5,770,429, the disclosures of all of which are incorporated by reference herein in their entirety.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Detection of the antibodies described herein may be achieved using standard ELISA, FACS analysis, and standard imaging techniques used in vitro or in vivo. Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, (3-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoeryethrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include 125I, 131I, 35S or 3H.

Particularly preferred, for ease of detection, is the sandwich assay, of which a number of variations exist, all of which are intended to be encompassed by the present invention. For example, in a typical forward assay, unlabeled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen binary complex, a second antibody, labeled with a reporter molecule capable of inducing a detectable signal, is added and incubated, allowing time sufficient for the formation of a ternary complex of antibody-antigen-labeled antibody. Any unreacted material
is then washed away, and the presence of the antigen is determined by observation of a signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include the simultaneous assay, in which both sample and antibody are added simultaneously to the bound antibody, or a reverse assay in which the labeled antibody and sample to be tested are first combined, incubated and added to the unlabeled surface bound antibody. These techniques are well known to those skilled in the art, and the possibility of minor variations will be readily apparent. As used herein, "sandwich assay" is intended to encompass all variations on the basic two-site technique. For the immunoassays of the present invention, the only limiting factor is that the labeled antibody be an antibody which is specific for the ESDN polypeptide or related regulatory protein, or fragments thereof.

The most commonly used reporter molecules are either enzymes, fluorophore- or radionuclide-containing molecules. In the case of an enzyme immunoassay an enzyme is conjugated to the second antibody, usually by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different ligation techniques exist, which are well-known to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, among others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for peroxidase conjugates, 1,2-phenylenediamine or toluidine are commonly used. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. A solution containing the appropriate substrate is then added to the tertiary complex. The substrate reacts with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an evaluation of the amount of polypeptide or polypeptide fragment of interest which is present in the serum sample.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labeled antibody absorbs the light energy, inducing a state of excitability in the molecule, followed by emission of the
light at a characteristic longer wavelength. The emission appears as a characteristic color visually detectable with a light microscope. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotopes, chemiluminescent or bioluminescent molecules may also be employed. It will be readily apparent to the skilled artisan how to vary the procedure to suit the required use.

The pharmaceutical compositions of the present invention may also comprise substances that inhibit the expression of ESDN at the nucleic acid level. Such molecules include ribozymes, antisense oligonucleotides, triple helix DNA, RNA aptamers, siRNA and/or double or single stranded RNA directed to an appropriate nucleotide sequence of ESDN nucleic acid. These inhibitory molecules may be created using conventional techniques by one of skill in the art without undue burden or experimentation. For example, modifications (e.g. inhibition) of gene expression can be obtained by designing antisense molecules, DNA or RNA, to the control regions of the genes encoding the polypeptides discussed herein, i.e. to promoters, enhancers, and introns. For example, oligonucleotides derived from the transcription initiation site, e.g., between positions -10 and +10 from the start site may be used. Notwithstanding, all regions of the gene may be used to design an antisense molecule in order to create those which gives strongest hybridization to the mRNA and such suitable antisense oligonucleotides may be produced and identified by standard assay procedures familiar to one of skill in the art.

Similarly, inhibition of the expression of gene expression may be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature (Gee, J.E. et al. (1994) In: Huber, B.E. and B. I. Carr, Molecular and Immunologic Approaches, Futura Publishing Co., Mt. Kisco, N.Y.). These molecules may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to inhibit gene expression by catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves
sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples which may be used include engineered "hammerhead" or "hairpin" motif ribozyme molecules that can be designed to specifically and efficiently catalyze endonucleolytic cleavage of gene sequences, for example, the gene for ESDN.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Ribozyme methods include exposing a cell to ribozymes or inducing expression in a cell of such small RNA ribozyme molecules (Grassi and Marini, 1996, Annals of Medicine 28: 499-510; Gibson, 1996, Cancer and Metastasis Reviews 15: 287-299). Intracellular expression of hammerhead and hairpin ribozymes targeted to mRNA corresponding to at least one of the genes discussed herein can be utilized to inhibit protein encoded by the gene.

Ribozymes can either be delivered directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression vector encoding the desired ribozymal RNA. Ribozymes can be routinely expressed in vivo in sufficient number to be catalytically effective in cleaving mRNA, and thereby modifying mRNA abundance in a cell (Cotten et al., 1989 EMBO J. 8:3861-3866). In particular, a ribozyme coding DNA sequence, designed according to conventional, well known rules and synthesized, for example, by standard phosphoramidite chemistry, can be ligated into a restriction enzyme site in the anticodon stem and loop of a gene encoding a tRNA, which can then be transformed into and expressed in a cell of interest by methods routine in the art. Preferably, an inducible promoter (e.g., a glucocorticoid or a tetracycline response element) is also introduced into this construct so that ribozyme expression can be selectively controlled. For saturating use, a highly and constitutently active promoter can be used. tDNA
genes (i.e., genes encoding tRNAs) are useful in this application because of their small size, high rate of transcription, and ubiquitous expression in different kinds of tissues.

Therefore, ribozymes can be routinely designed to cleave virtually any mRNA sequence, and a cell can be routinely transformed with DNA coding for such ribozyme sequences such that a controllable and catalytically effective amount of the ribozyme is expressed. Accordingly, the abundance of virtually any RNA species in a cell can be modified or perturbed.

Ribozyme sequences can be modified in essentially the same manner as described for antisense nucleotides, e.g., the ribozyme sequence can comprise a modified base moiety.

RNA aptamers can also be introduced into or expressed in a cell to modify RNA abundance or activity. RNA aptamers are specific RNA ligands for proteins, such as for Tat and Rev RNA (Good et al., 1997, Gene Therapy 4: 45-54) that can specifically inhibit their translation.

Gene specific inhibition of gene expression may also be achieved using conventional double or single stranded RNA technologies. A description of such technology may be found in WO 99/32619 which is hereby incorporated by reference in its entirety. In addition, siRNA technology has also proven useful as a means to inhibit gene expression (Cullen, BR Nat. Immunol. 2002 Jul;3(7):597-9; Martinez, J. et al., Cell 2002 Sept. 6;110(5):563).

Antisense molecules, triple helix DNA, RNA aptamers, siRNA, double or single stranded RNA and ribozymes of the present invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These methods include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the genes of the polypeptides discussed herein. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.
Vectors may be introduced into cells or tissues by many available means, and may be used in vivo, in vitro or ex vivo. For ex vivo therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection and by liposome injections may be achieved using methods that are well known in the art.

In addition to the above described methods for inhibiting the gene expression of ESDN, it is contemplated herein that one could identify and employ small molecules or other natural products to inhibit the transcription in vivo of the polypeptides discussed herein including, but not limited to, ESDN. For example, one of skill in the art could establish an assay for ESDN that can be easily applied to samples from the culture media of a cell line using conventional methods. Using this assay, cell lines would be screened to find ones that express ESDN. These cell lines would likely be of neuronal origin and would be cultured in, for example, 96 well plates. The closer the regulation of ESDN in the cell line to the expression in the dorsal root ganglia (DRG), the more likely it will be that small molecule modifiers of ESDN expression in the cell lines will also modify ESDN in DRG in vivo. A comparison of the effects of some known modifiers of gene expression e.g. dexamethasone, phorbol ester, heat shock on primary tissue DRG explants and the cell lines will allow the selection of the most appropriate cell line to use. The screen would then merely consist of culturing the cells for a set length of time with a different compound added to each well and then assaying for ESDN activity/ mRNA level.

In order to facilitate the detection of ESDN in the assay described above, luciferase or other commercially available fluorescent protein could be genetically fused as an appropriate marker protein to the promoter of ESDN. Sequences upstream of the ATG of ESDN, i.e. the promoter of ESDN, can be identified from genomic sequence data by using sequence information from GenBank to BLAST against the NCBI genomic sequence. This gives at least 5kb upstream of the ATG of ESDN that does not contain any unknown bases. Two pairs of nested PCR primers to amplify a fragment of 2 kb or longer from human genomic DNA can be readily designed and tested. The promoter fragment can be readily inserted into any promoter-less reporter gene vector designed for expression in human cells (e.g. Clontech promoter-less enhanced fluorescent protein vector pECFP-1, pEGFP-1, or pEYFP). The screen would then consist of culturing the cells for an appropriate length of time
with a different compound added to each well and then assaying for reporter gene activity. Promising compounds would then be assayed for effects on ESDN activity and/or mRNA level in vivo using the in vivo models of chronic pain previously described. Additional method details such as appropriate culturing time, culture conditions, reporter assays and other methodologies that can be used to identify small molecules or other natural products useful to inhibit the transcription of ESDN in vivo would be familiar to one of skill in the art.

In addition, the cDNA and/or protein of ESDN can be used to identify other proteins, e.g. receptors, that are modified by ESDN in neurons from DRG or other tissues in the nervous system. Proteins thus identified can be used for drug screening to treat chronic pain. To identify these genes that are downstream of ESDN, it is contemplated, for example, that one could use conventional methods to treat animals in chronic pain models with a specific ESDN inhibitor, sacrifice the animals, remove DRG and isolate total RNA from these cells and employ standard microarray assay technologies to identify message levels that are altered relative to a control animal (animal to whom no drug has been administered).

Based on the knowledge that ESDN is upregulated in chronic pain states, conventional in vitro or in vivo assays may be used to identify possible genes that lead to over expression of ESDN. These related regulatory proteins encoded by genes thus identified can be used to screen drugs that might be potent therapeutics for the treatment of chronic pain. For example, a conventional reporter gene assay could be used in which the promoter region of ESDN is placed upstream of a reporter gene, the construct transfected into a suitable neuronal cell (for example, a neuroblastoma cell line) and using conventional techniques, the cells assayed for an upstream gene that causes activation of the ESDN promoter by detection of the expression of the reporter gene.

It is contemplated herein that one can inhibit the function and/or expression of a gene for a related regulatory protein or protein modified by ESDN as a way to treat chronic pain by designing, for example, antibodies to these proteins and/or designing inhibitory antisense oligonucleotides, triple helix DNA, ribozymes, double or single stranded siRNA and RNA aptamers targeted to the genes for such proteins according to conventional methods. Pharmaceutical compositions comprising such inhibitory substances for the treatment of chronic pain are also contemplated.
The pharmaceutical compositions disclosed herein useful for treating and/or ameliorating chronic pain, including chronic neuropathic pain, are to be administered to a patient at therapeutically effective doses to treat or ameliorate symptoms of such disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of pain symptoms of chronic pain based on, for example, use of the McGill pain score (Melzack, R. Pain (1975) Sept. 1(3):277-299).

The inhibitory substances of the present invention can be administered as pharmaceutical compositions. Such pharmaceutical compositions for use in accordance with the present invention may be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or topical, oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.
Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulation agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic
materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC$_{50}$ (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms). Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamer, siRNA and double or single stranded RNA designed to inhibit ESDN gene expression, antibodies to ESDN or related regulatory proteins or fragments thereof, useful to treat and/or ameliorate the pathological effects of chronic pain. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED$_{50}$ (the dose therapeutically effective in 50% of the population) and LD$_{50}$ (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD$_{50}$/ED$_{50}$. Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage
contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc. Pharmaceutical formulations suitable for oral administration of proteins are described, e.g., in U.S. Patents 5,008,114; 5,505,962; 5,641,515; 5,681,811; 5,700,486; 5,766,633; 5,792,451; 5,853,748; 5,972,387; 5,976,569; and 6,051,581.

The following examples further illustrate the present invention and are not intended to limit the invention.
EXAMPLE 1
RNA Isolation and Expression Profiling of ESDN in Animal Models of Chronic Pain

In vivo animal models of chronic neuropathic pain include the following:

Seltzer Model.
In the Seltzer model (Seltzer et al. (1990) Pain 43: 205-218) rats are anaesthetised and a small incision made mid-way up one thigh (usually the left) to expose the sciatic nerve. The nerve is carefully cleared of surrounding connective tissues at a site near the trochanter just distal to the point at which the posterior biceps semitendinosus nerve branches off the common sciatic nerve. A 7-0 silk suture is inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle, and tightly ligated so that the dorsal 1/3 to 1/2 of the nerve thickness is held within the ligature. The muscle and skin are closed with sutures and clips and the wound dusted with antibiotic powder. In sham animals the sciatic nerve is exposed but not ligated and the wound closed as in nonsham animals.

Chronic Constriction Injury (CCI) model.
In the CCI model (Bennett, G.J. and Xie, Y.K. Pain (1988) 33: 87-107) rats are anaesthetised and a small incision is made mid-way up one thigh (usually the left) to expose the sciatic nerve. The nerve is cleared of surrounding connective tissue and four ligatures of 4/0 chromic gut are tied loosely around the nerve with approximately 1 mm between each, so that the ligatures just barely constrict the surface of the nerve. The wound is closed with sutures and clips as described above. In sham animals the sciatic nerve is exposed but not ligated and the wound closed as in nonsham animals.

Chung model.
In contrast to the Seltzer and CCI models which involves damage to peripheral nerves, the Chung model involves ligation of the spinal nerve. (Kim, S.O. and Chung, J.M. Pain (1992): 50:355-363). In this model, rats are anesthetized and placed into a prone position and an incision is made to the left of the spine at the L4-S2 level. A deep dissection through the paraspinal muscles and separation of the muscles from the spinal processes at
the L4-S2 level will reveal part of the sciatic nerve as it branches to form the L4, L5 and L6 spinal nerves. The L6 transverse process is carefully removed with a small rongeur enabling visualisation of these spinal nerves. The L5 spinal nerve is isolated and tightly ligated with 7-0 silk suture. The wound is closed with a single muscle suture (6-0 silk) and one or two skin closure clips and dusted with antibiotic powder. In sham animals the L5 nerve is exposed as before but not ligated and the wound closed as before.

Axotomy model.

The Axotomy model involves complete cut and ligation of the sciatic nerve. The nerve endings form neuromas but there is no behavioral correlate in this model as the nerve is not allowed to regenerate, and the foot is permanently denervated. (Kingery and Vallin, Pain 38, 321-32, 1989)

High Sciatic Lesion model.

In this model, the sciatic nerve is punctured in the region of the iliac arch. Although there is no overt damage to the nerve, local swelling produces an increase in pressure on the nerve as it passes under the iliac arch. This model resembles conditions often seen in the clinic.

Chronic inflammatory pain model:

The Complete Freund's Adjuvant-induced mechanical hyperalgesia may be used as a model of chronic inflammatory pain (Stein, C. et al. Pharmacol. Biochem. Behav. (1988) 31 :445-451). In this model, typically a male Sprague-Dawley or Wistar rat (200-250 g) receives an intraplantar injection of 25 μl complete Freund's adjuvant into one hind paw. A marked inflammation occurs in this hind paw. Drugs are generally administered for evaluation of efficacy, 24 hours after the inflammatory insult, when mechanical hyperalgesia is considered fully established.
Behavioral index

In all chronic pain models (inflammatory and neuropathic) mechanical hyperalgesia is assessed by measuring paw withdrawal thresholds of both hindpaws to an increasing pressure stimulus using an Analgesymeter (Ugo-Basile, Milan). Mechanical allodynia is assessed by measuring withdrawal thresholds to non-noxious mechanical stimuli applied with von Frey hairs to the plantar surface of both hindpaws. Thermal hyperalgesia is assessed by measuring withdrawal latencies to a noxious thermal stimulus applied to the underside of each hindpaw. With all models, mechanical hyperalgesia and allodynia and thermal hyperalgesia develop within 1 – 3 days following surgery and persist for at least 50 days. For the assays described herein, drugs may be applied before and after surgery to assess their effect on the development of hyperalgesia, particularly approximately 14 days following surgery, to determine their ability to reverse established hyperalgesia.

The percentage reversal of hyperalgesia is calculated as follows:

$$\% \text{ reversal} = \frac{\text{postdose threshold} - \text{predose threshold}}{\text{naive threshold} - \text{predose threshold}} \times 100$$

In the experiments disclosed herein, Wistar rats (male) are employed in the chronic neuropathic pain models described above. Rats weigh approximately 120-140 grams at the time of surgery. All surgery is performed under enflurane/O₂ inhalation anaesthesia. In all cases the wound is closed after the procedure and the animal allowed to recover.

In all but the axotomy model, a marked mechanical and thermal hyperalgesia and allodynia develops in which there is a lowering of pain threshold and an enhanced reflex withdrawal response of the hind-paw to touch, pressure or thermal stimuli. After surgery the animals also exhibit characteristic changes to the affected paw. In the majority of animals the toes of the affected hind paw are held together and the foot turned slightly to one side; in some rats the toes are also curled under. The gait of the ligated rats varies, but limping is uncommon. Some rats are seen to raise the affected hind paw from the cage floor and to demonstrate an unusual rigid extension of the hind limb when held. The rats tend to be very
sensitive to touch and may vocalise. Otherwise the general health and condition of the rats is good.

RNA extraction from DRG taken from rats subjected to chronic neuropathic pain models:

L4 and L5 DRG ipsilateral to the nerve injury are dissected from the rats at different times after surgery. Tissues are frozen in liquid nitrogen for subsequent total RNA preparation. Total RNA samples are prepared from the dissected DRG tissues according to the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi Anal. Biochem., (1987) 162:156-159; Chomczynski, P., Biotechniques, 15: 532-537 (1993)). The yield is approximately 1µg total RNA per DRG.

Sixteen RNA samples from rats subjected to the different chronic pain models were isolated at various time after surgery for Differential Display PCR (DD-PCR) analysis (Table 1).

<table>
<thead>
<tr>
<th>Samples #</th>
<th>Model</th>
<th>Days After Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Seltzer</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Seltzer</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>Seltzer</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>Seltzer</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>Sham</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Sham</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>Sham</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Sham</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>Axotomy</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>Chung (L4)</td>
<td>28</td>
</tr>
<tr>
<td>11</td>
<td>Chung (L5)</td>
<td>28</td>
</tr>
<tr>
<td>12</td>
<td>Chung Sham</td>
<td>28</td>
</tr>
<tr>
<td>13</td>
<td>CCI</td>
<td>14</td>
</tr>
<tr>
<td>14</td>
<td>CCI</td>
<td>21</td>
</tr>
<tr>
<td>15</td>
<td>High Sciatic</td>
<td>14</td>
</tr>
<tr>
<td>16</td>
<td>High Sciatic</td>
<td>21</td>
</tr>
</tbody>
</table>
DD-PCR, band isolation and subcloning:

The RNAimage™ mRNA Differential Display system from GenHunter Corp. (Nashville, TN) is used for DD-PCR analysis of the 16 RNA samples. The reverse transcription reaction is performed according to the manufacturer's protocol except a mixture of equal parts MMLV reverse transcriptase (GenHunter, Nashville, TN) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) is used. PCR amplification of the cDNA population is performed according to the manufacturer's instructions, using each of the 80 arbitrary primers with each one-base anchored oligo-dT primer and the similarly primed cDNA template. The PCR products are labelled with $^{35}$P-dATP and resolved on denaturing 6% urea-polyacrylamide gels with two sets of 16 PCR reactions loaded per gel. The combination of 80 rationally designed 13-mers and 3 one-base anchored oligo(dT)$_{11}$ allow 240 PCR reactions. Bands that are consistently up- or down-regulated in all the five models are isolated, re-amplified using the same primer pair used for DD-PCR and the PCR products subcloned into pCR4-TOPO vector through topoisomerase mediated TA cloning strategy (Invitrogen, Carlsbad, CA). Plasmid DNA is then prepared using QIAprep miniprep kit (QIAGEN, Valencia, CA) and sequenced using the T7 primer.

Confirmation of Regulated Genes by RT-PCR:

One ug of total RNA is treated with 0.1 unit of RNase-free DNase (Roche Mol.Biochem Indianapolis, IN,) at 37°C for 5 minutes and used for RT-PCR with primers designed from the sequences of the cloned cDNA fragments, according to conventional methods.

PCR products are analyzed on a 4-20% TBE Criterion polyacrylamide gel (BioRad, Hercules, CA,), stained with SYBR Green I (Molecular Probes, Eugene, OR) and visualized using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RACE –PCR:

Either the Marathon cDNA amplification kit (ClonTech, Palo Alto, CA) or the FirstChoice™ RLM-RACE kit (Ambion, Austin, TX) was used for PCR amplification of the cDNA sequences corresponding to bands of interest from rat total DRG RNA using protocols provided by the manufacturers. PCR products were cloned and sequenced as described above.
Example 2
Identification and Confirmation of Upregulated Transcripts in Multiple Pain Models

Examination of 240 sets of autoradiograms generated by the labeled DD-PCR products resulted in a total of 40 bands that are upregulated in at least two DRG RNA samples from the animal models, although no down-regulated products were identified. Among others, two of these bands, referred to as bands 28 and 31, are consistently upregulated in DRG samples from all 5 models. It was discovered that bands 28 and 31 are also derived from a single RNA species which had no sequence homology to anything in the GenBank databases. (Figure 1).

Gene-specific primers were designed from sequences of band 31 and used for RT-PCR confirmation from selected DRG total RNA samples. Data indicate that the gene has between 2-5 fold more RNA accumulation in DRG samples from rats in Chung, CCI, Seltzer and axotomy models, in agreement with the banding pattern on the original differential display gels.

Table 2. Identification of a gene upregulated in neuropathic pain models

<table>
<thead>
<tr>
<th>Band</th>
<th>Size (bp)</th>
<th>Identical Clones</th>
<th>RT-PCR Confirmation</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>183</td>
<td>4</td>
<td>Yes</td>
<td>Band 31</td>
</tr>
<tr>
<td>31</td>
<td>180</td>
<td>7</td>
<td>Yes</td>
<td>Band 31</td>
</tr>
</tbody>
</table>
Example 3

Molecular Cloning of Band 31

RACE-PCR was employed to amplify the longer cDNAs of band 31 in a step-wise fashion starting with a primer designed from the 3' end of the DD-PCR products, using total DRG RNA samples from animals of the Seltzer model at day 14. A distinct band of about 200 bp was observed at the third round of the RACE experiment, indicating we likely have reached the 5' end of the mRNA sequence. A contig of 2450 bases was generated from the sequences of the original DD-PCR product and PCR products of multiple rounds of RACE reactions (Figure 2). However, no obvious ORFs could be deduced from the nucleotide sequences. No sequences homologous to the contig were identified in GenBank or Incyte databases, although a LINE repeat is found in at its 3' portion. When clones of the 1.5 kb cDNA fragment from the second round of RACE PCR, which contains the most 5' end ATG start codons, were analyzed in an in vitro coupled transcription and translation, no detectable protein products larger than 5 kD were identified (data not shown). When the same cDNA fragment was labeled and hybridized to a multiple tissue Northern blot (Clontech, Palo Alto, CA) using conventional methods, mRNA species of 3.6 and 2.4 kb could be detected most abundantly in the brain sample, with the 2.4 kb species appearing also in liver, stomach, thymus and kidney (data not shown). However, not a single positive was identified from multiple plasmid or phage cDNA libraries from either rat DRG Seltzer day 14 or brain mRNAs, with a total of over 4 million primary clones screened using the RACE cDNA fragment as probes (data not shown). These results imply that the band 31 gene is expressed at low levels in brain and some other tissues.

Example 4

Band 31 Is 3' UTR of ESDN

In order to characterize band 31 using bioinformatics tools, the most 5' 650 bases of the 2.4 kb rat cDNA contig were used to search Celera assembled mouse genomic contigs (Celera, Rockville, MD). A 100 Kb mouse genomic DNA (CRA|GA_x5J8B7W8B52) was then retrieved and used to identify the corresponding human genomic DNA region (120 kb). For both the human and mouse genomic DNA sequences, there exist novel predicted transcripts
(hCT11214, mCT23485 and mCT23468). Two regions where human and mouse share significant homology were selected and primers for each region were designed based on the identical sequences between human and mouse and used for RT-PCR amplification of rat samples from Sham and Seltzer model at day 14. The amplified rat cDNA fragments of expected size were then cloned and sequenced according to conventional methods. New primers were subsequently designed based on the newly obtained rat sequences and used for secondary RT-PCR confirmation. About 3 fold upregulation was observed in Seltzer model compared to Sham at day 14 post operation. This implies that the predicted transcripts in human and mouse might be the human and mouse orthologs of rat band 31.

When the predicted transcripts (hCT11214, mCT23485 and mCT23468) and the newly cloned rat cDNA fragment as described above were used to search GenBank using BLAST protocol, three newly deposited cDNAs for endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN, human: AF387547; mouse: AF387548; rat: AF387549), were identified as the full-length cDNA transcripts, which was cloned by Kobuke et al (J. Biol. Chem. 276, 34105, 2001) using a yeast signal sequence trap (SST). ESDN encodes a 769 a.a. (mouse and rat) or 775 a.a. (human) type I transmembrane protein with a long (57 a. a.) signal peptide, followed by a CUB domain, a LCCL domain, a coagulation factor V/factor VII domain, a transmembrane domain and a long cytoplasmic domain composed of multiple potential phosphorylation sites. Its mRNA is up-regulated by vascular injury (balloon-injured carotid artery) and induced by PDGF in cultured vascular smooth muscle cells. It is also expressed abundantly in rodent nerve bundles. Because of the similarity of its domain structure to neuropilins, especially neuropilin 2, it is proposed that ESDN may have function in axon guidance. However, its unique long intercellular domain implies it may play a significant role in neuronal signal transduction.

To investigate whether ESDN is up regulated in neuropathic pain, two primers (forward: 5’-CCTGCGAGCCTGTGCTGCTTT-3’, SEQ ID NO 1) and (reverse 5’-GGTTGGTGGAAAGGAATGAAGCA-3’, SEQ ID NO 2) derived from the 3’ region of the rat ESDN (AF387549) are used for RT-PCR analysis and demonstrated that ESDN is upregulated 16 fold in the Seltzer model at day 14 (data not shown). This clearly indicates that ESDN is a useful new drug target for neuropathic pain.
EXAMPLE 5

Antisense Oligonucleotides to ESDN

Antisense oligonucleotides (ASOs) useful to inhibit gene expression, including the expression of ESDN, may be made according to conventional methods. In addition, one may employ additional methodologies, for example:

Synthesis of ASOs:

ASOs against ESDN may be fully phosphorothioated or fully phosphodiester 18-mers with nucleotides at both ends modified with MOE (methoxy ethoxy) groups. These may be synthesized using phosphoramidite chemistry, HPLC-purified and characterized by electrospray mass spectrometry and capillary gel electrophoresis according to conventional methods. ASOs, each with a GC content between 38 and 72%, may be selected and synthesized complementary to parts of the coding region of, for example, rat or human ESDN. For mismatch-containing control oligonucleotides, the approximate base composition of the match oligonucleotides may be maintained. Additionally, two control ASOs may be selected, e.g., one for rat GAPDH coding regions and a second random synthetic ASO. The format of the anti-rat-GAPDH oligonucleotide may be the same as for anti-ESDN oligonucleotides; the synthetic oligonucleotide may have its MOE ribonucleotide modifications at both ends of the sequence with phosphorothioate or phosphodiester DNA residues in the middle.

Transfection protocol:

Twenty four hours before transfection, 2 x 10^5 cells e.g., Chinese Hamster Ovary cells (ICN Pharmaceuticals Ltd., Basingstoke, Hampshire, U.K.) in a volume of 2 ml per well (F12 Nutrient mix (DMEM), 100 unit/ml Penicillin, 100 micrograms per millilitre streptomycin, 2 millimolar L-Glutamine, 10% fetal bovine serum (GIBCO-BRL, Rockville, MD)) may be plated into 6-well plates and cultured in 5% CO_2 to yield 70-80% confluency. On the day of transfection, a 2 fold stock transfection solution is prepared by diluting Lipofectin™ into serum-free OptiMEM (GIBCO-BRL, Rockville, MD) (3 microliters Lipofectin™ per 100 nM desired final oligonucleotide concentration into 1ml OptiMEM) and incubating for 15 minutes at room temperature. This solution is then combined 1:1 with a 2 fold ASO-solution containing twice the desired final amount of ASO in OptiMEM. After incubating the
transfection mixture for 15 minutes at room temperature to form the transfection complex, 2 ml is added to each of the previously aspirated well of cells. A Lipofectin™ reagent-only control and a normal cell control (untreated) may also be included. After incubation for 4 hours at 37°C, 500 microlitres of 50% FBS in MEM (GIBCO-BRL) is then added to each well to obtain a final FBS concentration of 10%. The cultures are then incubated at 37°C in a humidified incubator with 5% CO₂ for 24 hours for mRNA harvest or 48 hours for protein harvest and electrophysiology.

Real-time quantitative PCR mRNA analysis:

Total RNA may be isolated with the RNeasy 96 Kit (Qiagen, GmbH, Germany) according to the manufacturer's protocol. The RNA samples are individually diluted to 1ng/L. Five nanograms of RNA for each sample are then mixed with gene-specific detection primers (easily determined by one of skill in the art) and with the appropriate reagents from the real-time quantitative PCR reaction kit PLATINUM® Quantitative RT-PCR THERMOSCRIPT™ One-Step System (Gibco-BRL, Rockville, MD) and run according to manufacturer's protocol. The rat ESDN primers with the appropriate sequences may be easily designed by one of skill in the art and purchased from commercial vendors (for example, Sigma Chemical, St. Louis, MO). GAPDH or beta-actin may be chosen as a control gene for comparisons. The same RNA samples may be run with rat GAPDH primers from the TaqMan® Rodent GAPDH Control Reagents Kit (PE Biosystems). The sequence-specific fluorescent emission signal can be detected using the ABI PRISM™ 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA). Along with the samples, a standard from dilutions of pure template mRNA is run to obtain absolute concentrations per inserted amount of total RNA.

Testing the ESDN antisense in animal models of neuropathic pain:

Rats (e.g. Wistar) may be intrathecally cannulated in the lumbar or thoracic region of the spinal cord with a catheter attached to a minipump delivery system according to conventional methods. Antisense, missense oligos or vehicle may then be delivered for up to 7 days at a desired concentration to allow cell bodies within the spinal cord and the dorsal root ganglia to take up the oligos or vehicle. Nerve injury may be performed either before or after cannulation according to the pain models described herein. Mechanical hyperalgesia,
alldynia etc may be measured in the usual way to assess the effect of ESDN antisense oligonucleotides in reversal of hyperalgesia.

EXAMPLE 6

siRNA Oligonucleotides to ESDN

Short inhibitory RNAs (siRNA) useful to inhibit gene expression, including the expression of ESDN, may be made according to conventional methods. In addition, one may employ additional methodologies, for example:

Synthesis of Oligoribonucleotides (siRNA's)

Modified synthetic oligoribonucleotides described herein can be prepared using standard TOM-phosphoramidite chemistry on ABI394 or Expedite/Moss Synthesizers (Applied Biosystems) for in vitro use and on OligoPilot II (Amersham Pharmacia Biotech) for in vivo purposes. Phosphoramidites are dissolved in acetonitrile at 0.05 M concentration (0.2M on Oligopilot II), coupling is made by activation of phosphoramidites by a 0.2 M solution of benzimidazolium triflate in acetonitrile. Coupling times are usually comprised between 3-6 minutes. A first capping is made using standard capping reagents. Sulfurization is made by using a 0.05 M solution of N-ethyl, N-phenyl-5-amino-1,2,4-dithiazol-3-thione for two minutes (described in EP-A-0992506). Oxidation is made by a 0.1 M iodine solution in THF/Pyridine/Water (77:20:3) or 0.5M t-butylhydroperoxide (Fluka) in dichloromethane for two minutes. A second capping is performed after oxidation or sulfurization. Oligonucleotide growing chains are detritylated for the next coupling by 2% dichloroacetic acid in dichloromethane or dichloroethane. After completion of the sequences the support-bound compounds are cleaved and deprotected as "Trityl-on" by a Methylamine solution (41% aqueous methylamine/33% ethanolic methylamine 1:1 v/v) at 35ºC for 6 h. Resulting suspensions are lyophilised to dryness. 2'-O-silyl groups are removed upon treatment with 1M tetrabutylammonium fluoride 10min at 50ºC and 6h at 35ºC. The obtained crude solutions are directly purified by RP-HPLC. The purified detritylated compounds are analysed by Electrospray Mass spectrometry and Capillary Gel Electrophoresis and quantified by UV according to their extinction coefficient at 260 nM.
Generation of a cell lines expressing rat ESDN (ESDN-CHO)

Chinese hamster ovary cells (CHO-K1, ATCC CCL61) can be stably transfected with a complete rat ESDN cDNA sequence. To be able to select for transfected cells, vector can be co-electroporated in 10x excess with pMC1neo (Stratagene, La Jolla, CA) containing a Neomycin resistance gene. Cells can be cultured in Minimal Essential Medium α (MEMα) supplemented with 10% (v/v) fetal bovine serum (FBS), 2-mM glutamine, and 10,000 IU/500 ml Penicillin/Streptomycin.

Electroporation of Mammalian Cells

CHO cells can be transfected with 0.15; 0.3; 0.6 or 1.2 nmole of siRNA duplex using standard electroporation (10⁶ cells/125 μl in Biorad cuvette 0.4 cm, 250V, 0.3 μF, infinite resistance). Following electroporation, samples can be immediately combined with 6 μl of the culture medium. Cells can then be plated on uncoated 96-well plates (Costar, Cat. #3904) and incubated at 37°C for 24h or 48h, followed by RNA or protein extraction, respectively.

Transfection protocol: Oligofectamine Treatment of Mammalian Cells.

siRNA duplexes, 20 μM in hybridization buffer (100 mM KAc, 30 mM Hepes-KOH pH 7.4, 2 mM MgAc) can be transfected into cells on 24-well plates: 5 × 10⁴ ESDN expressing CHO-K1 cells (or an equivalent ESDN expressing cell line)/well in 0.5ml MEMα+ 10% FCS without antibiotics through use of Oligofectamine (Invitrogen # 12252011: 1 ml).

RNA oligonucleotides can be hybridized by preparing a mix of both ssRNA oligonucleotides at 20 μM in Hyb buffer followed by incubation for 1 min at 90°C followed by 1 hr at 37°C.

For an siRNA concentration of 200 nM (based on a 300 μl total volume on cells) per well, in a 24-well plate format following solutions can be formulated:

A: 50 μl Optimem + 3 μl of 20 μM siRNA duplex, vortex.
B: 12 μl Optimem + 3 μl oligofectamine, mix gently, incubate 7-10’ RT.
Add B to A (68 μl), mix gently, incubate 20-25’ RT.
Add 38 μl Optimem (106 μl volume), mix gently.
After aspiration of medium from plate, it can then be washed once with Optimem, and 200 μl Optimem added per well.
The cells can be overlaid with the 106 μl complex, further incubated 4 hrs at 37°C.
Add 300 µl Optimem and 70 µl serum, incubate 24-48 hrs. The cultures can then be incubated at 37°C in a humidified incubator with 5% CO₂ for 24 hours for mRNA harvest or 48 hours for protein harvest and electrophysiology.

Total RNA Isolation and Assay by Quantitative Real Time PCR (Q-PCR)

Total RNA can be extracted and purified using RNeasy 96 kit (Qiagen, Valencia CA). Primer pairs and FAM-labelled TaqMan probes for real time PCR can be designed using the Primer Express v 2.0 program (ABI PRISM, PE Biosystems). For the Q-PCR reaction, 50 ng total RNA can be mixed with 5' and 3' primers (10 µM each), TaqMan probe (5 µM), MuLV reverse transcriptase (6.25 u, PE Biosystems), RNase Out RNase inhibitor (10 u, Life Technologies) and the components of the TaqMan PCR reagent kit (Eurogentec) in a total volume of 25 µl following the TaqMan PCR reagent kit protocol (Eurogentec). Reverse transcription and real time PCR can be performed in a GeneAmp Sequence Detector 5700 (PE Biosystems) as follows: 2 minutes reverse transcription at 50°C, 10 minutes denaturation at 95°C followed by 50 cycles of denaturation for 15 sec at 95°C and annealing and elongation for 1 min at 60°C. The relative quantitation of gene expression can be calculated as described in the ABI PRISM 7700 user bulletin #2 (PE Biosystems).

Western Blotting

Cells grown in 6-well plates can be washed with PBS and lysed with a buffer containing 141 mM NaCl, 5 mM KCl, 2.5 mM Tris pH 7.4, 50 mM Va3VO4, 0.1% (v/v) Nonidet P-40 (100%), and 0.06 g protease inhibitor per 100 ml. Lysates can be centrifuged for 10 min at 14000 rpm. Solubilized proteins in the supernatant can be subjected to SDS-polyacrylamide gel electrophoresis through NuPAGE™ 4-12% Bis-Tris Gels in a NOVEX™ Mini-Cell system, followed by transfer to PVDF membranes (Millipore). The filters can then be blocked for 1h with the blocking buffer contained in the ECF Western Blotting Kit (Amersham Pharmacia Biotech), washed several times in 1x PBS, pH 7.4 with 0.05% Tween 20, and incubated for 1h with the primary specific anti-antibody in an appropriate dilution (for example: 1:5000). With several washes in between, the filters can then be incubated with the secondary antibody, tertiary antibody and ECF substrate from the ECF Western Blotting Kit following the manufacturer's suggestions. A quantification of the visualized bands can be done with the software ImageQuant™ (Molecular Dynamics).
FLIPR Assay - Generation and Analysis of FLIPR Data

FLIPR experiments, or equivalent functional assays, can then be performed as follows. Briefly, cells can be loaded with fluo-4 AM in presence of 2.5 mM probenecid for 30-45 min, washed twice with HBSS (Gibco) + 20mM HEPES, and transferred to a fluorescence reader (FLIPR, Molecular Devices). Drug plates can then be prepared at 5x the final concentration. Fluo-4 fluorescence can be measured at a rate of 0.5 Hz for 3 min. Agonists can be applied after 20 points baseline detection.

FLIPR sequence files can be analyzed using Igor Pro (Wavemetrics). Baseline can be set as the average of 20 points before drug addition, peak is detected as maximal signal in the 50 data points after drug addition. Relative change of fluorescence (dF/F) may be determined as (peak – baseline) / (baseline) values. These values can then be averaged, and for concentration-response analysis further analyzed by fitting a sigmoidal hill equation to the data. Data can be presented as mean +/- S.E.M. or EC50 values as mean (95% confidence interval).

Real-time quantitative PCR mRNA analysis:

Total RNA may be isolated with the RNeasy 96 Kit (Qiagen, GmBH, Germany) according to the manufacturer's protocol. The RNA samples are individually diluted to 1ng/L. Five nanograms of RNA for each sample are then mixed with gene-specific detection primers (easily determined by one of skill in the art) and with the appropriate reagents from the real-time quantitative PCR reaction kit PLATINUM® Quantitative RT-PCR THERMOScript™ One-Step System (Gibco-BRL, Rockville, MD) and run according to manufacturer’s protocol. The rat ESDN primers with the appropriate sequences may be designed and purchased. GAPDH or beta-actin may be chosen as a control gene for comparisons. The same RNA samples may be run with rat GAPDH primers from the TaqMan® Rodent GAPDH Control Reagents Kit (PE Biosystems). The sequence-specific fluorescent emission signal can be detected using the ABI PRISM™ 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA). Along with the samples, a standard from dilutions of pure template mRNA is run to obtain absolute concentrations per inserted amount of total RNA.

Testing the ESDN antisense and/or siRNA in animal models of neuropathic pain:
Rats (e.g. Wistar) may be intrathecally cannulated in the lumbar or thoracic region of the spinal cord with a catheter attached to a minipump delivery system according to conventional methods. Antisense, missense oligos, siRNA or vehicle may then be delivered for up to 7 days at a desired concentration to allow cell bodies within the spinal cord and the dorsal root ganglia to take up the oligos or vehicle. Nerve injury may be performed either before or after cannulation according to the pain models described herein. Mechanical hyperalgesia, alldynia etc may be measured in the usual way to assess the effect of ESDN antisense and/or siRNA oligonucleotides in reversal of hyperalgesia.
What is claimed is:

1. A method to treat or ameliorate chronic pain comprising administering to a subject in need thereof an effective amount of a ESDN modulator.

2. The method of claim 1 wherein said chronic pain is chronic neuropathic pain.

3. The method of claim 1 wherein said ESDN modulator inhibits the activity of ESDN in said subject.

4. The method of claim 1 wherein said ESDN modulator inhibits ESDN gene expression in said subject.

5. The method of claim 1 wherein said modulator comprises any one or more substances selected from the group consisting of antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamers, siRNA and double or single stranded RNA wherein said substances are designed to inhibit ESDN gene expression.

6. The method of claim 1 wherein said modulator comprises one or more antibodies to ESDN, or fragments thereof, wherein said antibodies or fragments thereof can inhibit ESDN activity.

7. A method to treat or ameliorate chronic pain comprising administering to a subject in need thereof a pharmaceutical composition comprising an effective amount of a ESDN modulator.

8. The method of claim 7 wherein said chronic pain is chronic neuropathic pain.

9. The method of claim 7 wherein said ESDN modulator inhibits the activity of ESDN in said subject.

10. The method of claim 7 wherein said ESDN modulator inhibits ESDN gene expression in said subject.
11. The method of claim 7 wherein said modulator comprises any one or more substances selected from the group consisting of antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamers, siRNA and double or single stranded RNA wherein said substances are designed to inhibit ESDN gene expression.

12. The method of claim 7 wherein said modulator comprises one or more antibodies to ESDN, or fragments thereof, wherein said antibodies or fragments thereof can inhibit ESDN activity.

13. A method to identify modulators useful to treat or ameliorate chronic pain comprising assaying for the ability of a candidate modulator to inhibit ESDN activity.

14. The method of claim 13 wherein said method further comprises assaying for the ability of an identified ESDN inhibitory modulator to reverse the pathological effects observed in animal models of chronic pain and/or in clinical studies with subjects with chronic pain.

15. The method according to claim 13 wherein said chronic pain is chronic neuropathic pain.

16. A method to identify modulators useful to treat or ameliorate chronic pain comprising assaying for the ability of a candidate modulator to inhibit ESDN gene expression.

17. The method according to claim 16 wherein said method further comprises assaying for the ability of an identified inhibitory modulator to reverse the pathological effects observed in animal models of chronic pain and/or in clinical studies with subjects with chronic pain.

18. The method according to claim 16 wherein said chronic pain is chronic neuropathic pain.

19. A pharmaceutical composition comprising a ESDN modulator in an amount effective to treat or ameliorate chronic pain in a subject in need thereof.

20. The pharmaceutical composition according to claim 19 wherein said chronic pain is chronic neuropathic pain.
21. The pharmaceutical composition according to claim 19 wherein said modulator inhibits the activity of ESDN.

22. The pharmaceutical composition according to claim 19 wherein said modulator inhibits ESDN gene expression.

23. The pharmaceutical composition of claim 19 wherein said modulator comprises any one or more substances selected from the group consisting of antisense oligonucleotides, triple helix DNA, ribozymes, RNA aptamer, siRNA and double or single stranded RNA wherein said substances are designed to inhibit ESDN gene expression.

24. The pharmaceutical composition of claim 19 wherein said modulator comprises one or more antibodies to ESDN, or fragments thereof, wherein said antibodies or fragments thereof can inhibit ESDN activity.

25. A method to diagnose subjects suffering from chronic pain who may be suitable candidates for treatment with ESDN modulators comprising assaying mRNA levels of said protein in a biological sample from said subject wherein subjects with increased levels compared to controls would be suitable candidates for ESDN modulator treatment.

26. A method to diagnose subjects suffering from chronic pain who may be suitable candidates for treatment with ESDN modulators comprising detecting levels of said protein in a biological sample from said subject wherein subjects with increased levels compared to controls would be suitable candidates for ESDN modulator treatment.

27. A method to treat or ameliorate chronic pain comprising:

(a) assaying for ESDN mRNA and/or protein levels in a subject; and,
(b) administering to a subject with increased levels of ESDN mRNA and/or protein levels compared to controls an ESDN modulator in an amount sufficient to treat or ameliorate the pathological effects of chronic pain.
28. The method of claim 27 wherein said chronic pain is chronic neuropathic pain.

29. A diagnostic kit for detecting mRNA levels and/or protein levels of ESDN in a biological sample, said kit comprising:

(a) a polynucleotide of ESDN or a fragment thereof;
(b) a nucleotide sequence complementary to that of (a);
(c) an RNAi sequence complementary to that of (a);
(d) a ESDN polypeptide, or a fragment thereof; or
(e) an antibody to a ESDN polypeptide

wherein components (a), (b), (c), (d) or (e) may comprise a substantial component.
Figure 1.

TTTTTTTTTT TCAGGGTTT GCAGAGGATT TTAAAGAGGA GTAACAGGGG
GTTCCCACCC ATGCCATTTG TAACCCATGC TACACATCAT CTCTGTTTGT
TATTACATGA ATTCACCTTG ACTGCT AATG AGTACAGACC CTCCATCCCAT
GGTAGCCTCC CACACTTTCT GCACATAGCC AA

SEQ ID NO 3
Figure 2 A.

3rd RACE (229 bp)

2nd RACE (1513 bp)

1st RACE (1010 bp)

ED-PCR Product (378 bp)

Contig Sequence: 2450 bp

5' 1 44 229 1440 1556 2273 2460 3'