# SODIUM CHANNEL REGULATORS AND MODULATORS

## Field of the Invention

The present invention relates generally to methods and materials for use in regulating or modulating voltage gated $Na^+$ channels (VGSCs).

## Background of the Invention

VGSCs are transmembrane proteins responsible for bestowing electrical excitability upon almost all excitable membranes. The pore is gated by depolarization of the cell membrane, transiently allowing $Na^+$ ions to enter into the cell, and generating the upswing of an action potential. Following activation, VGSCs undergo inactivation, limiting the action potential duration, and allowing rapid membrane repolarization followed by a return to the resting state. All known VGSCs exhibit remarkable functional similarities and this is reflected in a high degree of amino-acid sequence homology. However, natural toxins are known to discriminate well between $Na^+$ channel subtypes. For example, tetrodotoxin (TTX) from the Puffer fish, can selectively block subtypes of neuronal VGSCs at single nanomolar concentrations, whereas other neuronal VGSCs remain unblocked by the toxin at micromolar concentrations. These neuronal VGSCs that are TTX-insensitive or resistant (TTX-R) are found in the peripheral nervous system, and are exclusively associated with nerves involved in the transmission of pain (see e.g. Akopian et al (1999) "The tetrodotoxin-resistant sodium channel SNS plays a specialised role in pain pathways". Nature Neuroscience 2, 541-548).

WO 97/01577 (University College London) relates to a novel 1,957 amino acid TTX-insensitive VGSC from mammalian sensory neurons (which has been designated Nav 1.8). US 6184349 (Syntex) discusses VGSCs. The sodium channel Nav1.8 (also known as SNS or PN3) is expressed exclusively in small diameter sensory neurones that correspond to Aδ or C-fibre nociceptors, which are the cells

that transmit pain signals. One key feature of Nav1.8 pharmacology is its resistance to high concentrations of tetrodotoxin (TTX), which blocks most other sodium channels. Evidence for a role of Nav1.8 in pain signalling comes largely from knock out mice and from studies where the channel is downregulated with antisense oligonucleotides. These experiments suggest that Nav1.8 is important in models of inflammatory, neuropathic and visceral pain.

Nav1.9 (SNS2) is also found exclusively in sensory neurones that signal pain and is also resistant to TTX. The properties of the channel suggest that it is not involved in generation or propagation of action potentials but is involved in setting the level of excitability of the cell. There is evidence that G-proteins can activate Nav1.9, which in turn increases neuronal excitability and makes the cell more likely to fire. There is no direct evidence for involvement of Nav1.9 in pain models, but given its function in the cell and the restricted distribution, it could play a major role in producing the hyper-reactivity associated with many chronic pain states.

Nav 1.3 is found in brains of adult animals and is sensitive to TTX. There is normally no Nav1.3 in sensory neurones, but after nerve damage, levels are upregulated massively. Again there is no direct evidence for involvement of Nav1.3 in pain, but the selective upregulation after nerve injury suggests that it might play a role in transmission of neuropathic pain signals.

p11 is a member of the S-100 family small calcium binding proteins. p11 is also known as annexin-II light chain, lipocortin-II light chain, calpactin I light chain, 42C, or S-100 related protein, and these terms may be used interchangeably herein. It is present in a variety of cells separately or as a heterotetramer. The heterotetramer is composed of two copies of p36, also known as annexin-II or calpactin-I heavy chain, and two copies of p11. The association of p11 with p36 is $Ca^{2+}$-independent and of high affinity, and binding of p11 to p36 strongly enhances p36's ability to bundle F-actin. Within the cell, the heterotetramer is localized at the cytoplasmic surface of the plasma membrane in the submembranous cytoskeleton, and it is suggested that

the complex may play a role in membrane trafficking events such as exocytosis, endocytosis and cell-cell adhesion. It is also known that p11 is a strong substrate for tyrosine kinase pp60src and its phosphorylation is a negative modulator of tetramerization and protein function.

**Summary of the Invention**

The present invention derived from the Inventors' finding that the p11 protein is involved in the functional expression of voltage gated sodium channels (VGSCs). The present invention provides screening methods for the identification of compounds which are capable of modulating VGSCs. In one aspect there is provided a method of identifying a modulator of a VGSC, which method comprises:

(a) bringing into contact a VGSC, a p11 peptide and a test compound under conditions where the VGSC and the p11 peptide are capable of forming a complex in the absence of the test compound; and

(b) measuring an activity of the VGSC, wherein a change in the activity of the VGSC relative to the activity in the absence of the test compound indicates that the test compound is a modulator of said VGSC.

The invention further provides a method of enhancing the functional expression of a voltage gated sodium channel (VGSC) in a cell which method comprises the step of increasing the level of p11 in the cell.

The present invention also provides a host cell capable of expressing a VGSC and a p11 peptide wherein said VGSC and/or said p11 peptide is expressed from one or more heterologous expression vectors within said cell. Such a host cell may be used in the screening methods of the invention.

The invention also provides compounds identified as putative modulators of VGSC activity by the screening methods of the invention. Such compounds may be used in the treatment or disorders which involve VGSCs. The invention therefore provides

the use of a compound identified by a screening method of the invention in the manufacture of a medicament for modulating the functional expression of a voltage gated sodium channel. Also provided is a method of treating a disorder or condition associated with the activity of a voltage gated sodium channel, said method comprising administering to an individual in need thereof a compound identified by a screening method of the invention.

Also within the scope of the invention are methods of decreasing VGSC activity or functional expression by decreasing the levels of p11. The present invention therefore provides the use of an inhibitor of p11 activity or expression in the manufacture of a medicament for modulating the functional expression of a voltage gated sodium channel. Also provided is a method of treating a disorder or condition associated with the activity of a voltage gated sodium channel, said method comprising administering to an individual in need thereof an inhibitor of p11 activity or expression.

The invention also provides isolated peptides derived from VGSCs and p11 which comprise the amino acids involved in binding between these two proteins. There is therefore provided a peptide comprising at least 10 contiguous amino acids of: (a) the sequence from amino acid 75 to amino acid 102 of SEQ ID NO: 2, or (b) a sequence having at least 65% amino acid sequence identity to (a); wherein said peptide is capable of specifically binding a p11 peptide and is less than 1000 amino acids in length. There is also provided a peptide comprising at least 10 contiguous amino acids of: (a) the sequence from amino acid 33 to amino acid 77 of SEQ ID NO: 4, or (b) a sequence having at least 70% amino acid sequence identity to (a); wherein said peptide is capable of specifically binding a voltage gated sodium channel and is less than 80 amino acids in length.

**Brief Description of the Drawings**

Figure 1A: High threshold TTX-resistant $Na^+$ current recorded from fluorescent

CHO-SNS22 cells after transfection with GFP-p11 cDNA expression vector. $Na^+$ current has characteristically slow kinetics, and inward current is abolished by removing extracellular $Na^+$ ions. Pulse protocol is shown above.

Figure 1B: Average current ($I/I_{max}$) – voltage ($E_m$) relationship for the $Na^+$ current in CHO-SNS22 cells (n=5). The threshold for activation is close to –5mV and the current peaks at +40mV.

Figure 2: p11 antisense mRNA expression in DRG neurons caused a loss of Nav1.8 current density. The histogram shows cell number against log[current density] for control and cDNA-injected neurons. White bars = non-injected control cells; grey bars = GFP-injected cells; Black bars = p11 antisense and GFP-injected cells. Two-tailed unpaired t-test for the log[current density] of neurons injected with GFP expression vector only and p11 antisense mRNA expression vector showed significant reduction in Nav1.8 current ($P < 0.02$, Student's two-tailed t-test).

**Brief Description of the Sequences**

SEQ ID NO: 1 is the DNA sequence of the rat Nav 1.8 receptor gene and SEQ ID NO: 2 is the amino acid sequence that it encodes. These sequences are publicly available from GenBank under accession number X92184.

SEQ ID NO: 3 is the DNA sequence of the rat p11 gene and SEQ ID NO: 4 is the amino acid sequence that it encodes. These sequences are publicly available from GenBank under accession number J03627.

SEQ ID NO: 5 is the DNA sequence of the human Nav 1.8 receptor gene and SEQ ID NO: 6 is the amino acid sequence that it encodes. These sequences are publicly available from GenBank under accession number AF117907.

SEQ ID NO: 7 is the DNA sequence of the human p11 gene and SEQ ID NO: 8 is the amino acid sequence that it encodes. These sequences are publicly available from GenBank under accession number NM_002966.

## Detailed Description of the Invention

The present invention relates generally to screening methods for the identification of compounds capable of regulating or modulating the functional expression of sodium channels. Also provided are methods wherein such compounds are used in the treatment of conditions associated with sodium channel function, for example in the prevention or treatment of pain.

As described in more detail below, this interaction may be exploited, *inter* alia, in:

(i) enhancing the functional expression of a sodium channel e.g. in cell lines which may be used for conventional modulator-screening purposes,

(ii) defining a novel target (i.e. disruption of the protein-protein interaction site itself) for devising modulators which could lower the functional expression of a sodium channel.

*Sodium channels and p11 peptides*

The present application relates to the regulation or modulation of functional expression of sodium channels, in particular voltage gated sodium channels (VGSCs). Table 1 indicates the sequence identity between various VGSC molecules, using the rat Nav 1.8 channel as a basis for comparison:

**Table 1**

| Channel | Rat 1.8 | Rat 1.5 | Rat 1.9 | Rat 1.3 |
|---|---|---|---|---|
| Accession number | X92184 | M27902 | AF059030 | Y00766 |
| With gaps | 100 | 61% | 49% | 57% |
| Without gaps | 100 | 63% | 55% | 62% |

For comparison, rat 1.8 vs human 1.8 scores 83% (with gaps) or 84% (without gaps) identity using this method

Amino acid identity was determined over the full protein sequence. The Nav1.8 protein sequence was aligned with a second sequence using Clustal. The number of identical amino acids was then scored for each pair and divided by the total number of amino acids in the alignment (with gaps) or the total number of aligned amino acids (without gaps).

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A VGSC of the invention is any VGSC which has the ability to specifically bind a p11 peptide. By specifically bind it is meant that the VGSC binds the p11 peptide preferentially to a non-p11 peptide, for example a VGSC binds more straongly to a p11 peptide than to a randomly generated non-p11 peptide sequence.

In particular, the present invention relates to VGSCs that are associated with responses to pain or are involved in pain signalling. A suitable sodium channel is preferably a VGSC that is expressed in sensory neurons. For example, a suitable VGSC may be a sensory neuron specific (SNS) VGSC, for example Nav 1.8 or Nav 1.9, or may be upregulated in sensory neurons in response to pain, for example Nav 1.3. A suitable VGSC may be tetrodotoxin (TTX) insensitive or resistant, that is, it may remain unblocked by TTX at micromolar concentrations.

In one aspect, a VGSC for use in methods of the invention is a Nav 1.8, Nav 1.9 or Nav 1.3 channel. The nucleotide and amino acid sequences for the Nav 1.8, rat Nav 1.9 and rat Nav 1.3 channels are publicly available, for example rat sequences are available from GenBank under the accession numbers given in Table 1. The nucleotide and amino acid sequences for rat Nav 1.8 are given in SEQ ID Nos: 1 and 2 respectively and the nucleotide and amino acid sequences for human Nav 1.8 are given in SEQ ID Nos: 5 and 6 respectively.

A suitable VGSC for use in the methods of the invention may be any of these VGSCs or a species or allelic variant of any thereof. There is no requirement that the proteins (or nucleic acids) employed in the present invention have to include the full-length

"authentic" sequence of the proteins as they occur in nature. A suitable VGSC may therefore also be a variant of any of these VGSCs which retains activity as a sodium channel. For example, a suitable VGSC may have greater than 65%, greater than 70%, greater than 75%, greater than 85%, greater than 95% or greater than 98% amino acid identity with any of the Nav 1.8, Nav 1.9 or Nav 1.3 sequences.

A suitable variant channel is one which retains sodium channel function. For example, a suitable variant of the Nav 1.8 sodium channel may have the normal function of a VGSC. The function of a VGSC may be measured as described below. It may also retain the tetrodotoxin insensitivity of the Nav 1.8 channel.

A suitable variant preferably also retains the ability to bind p11. For example, a suitable variant channel may retain the intracellular domain of a wild type VGSC. For example, a preferred variant of the rat Nav 1.8 channel may retain the N-terminal intracellular domain found at positions 1 to 127 of SEQ ID NO: 2. A suitable variant channel may have a sequence comprising amino acids 53 to 127 or amino acids 75 to 102 of SEQ ID NO: 2, which are shown below to be involved in binding to p11 protein, or a species or allelic variant of this region.

A suitable variant VGSC may be a fragment of a wild type VGSC or of a variant thereof as described above. A suitable fragment may be a truncated VGSC, wherein, for example, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 50% or more of the original VGSC sequence has been removed. A suitable fragment may consist of or comprise a fragment of a full length VGSC, for example, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 50% or more of a full length sequence. A suitable fragment may be any fragment which retains the ability to bind a p11 peptide. A suitable fragment may also retain the ability to function as a sodium channel. A fragment may be, for example, 10, 20, 30, 50, 75, 100, 150, 200, 300, 500, 750, 1000, 1500 or more amino acids in length.

A suitable VGSC may comprise a fragment of a wild-type or variant VGSC sequence as part of its amino acid sequence. Such a variant will retain the ability to bind p11,

and optionally the ability to act as a sodium channel. A VGSC fragment which retains the ability to bind p11 may be derived from the intracellular domain of the full-length VGSC. Such a fragment may include the entire intracellular domain or a part thereof. A preferred fragment of the Nav 1.8 channel may be selected from the N-terminal intracellular domain, for example amino acids 1 to 127 of SEQ ID NO: 2. Preferably fragments represent sequences which are believed to be either unique to the channel, or are at least well conserved among VGSCs. Preferred fragments of SEQ ID NO: 2 include amino acid positions 1 to 25, 26 to 50 and 51 to 127. A VGSC fragment which retains the ability to bind p11 may consist of or comprise the sequence of amino acids 53 to 127 or 75 to 102 of SEQ ID NO: 2. Such a VGSC fragment may be, for example, 28 to 50, 28 to 100, 28 to 200, 28 to 500, 28 to 1000 amino acids in length or larger. A suitable VGSC fragment may comprise a part of the sequence of amino acids 53 to 127 or 75 to 102, for example, 5, 10, 15, 20, or 25 contiguous amino acids from this region or from a variant of this region as defined above, which retain the ability to bind p11.

Thus, in one aspect there is provided a peptide comprising at least 10, at least 15, at least 20 or at least 25 contiguous amino acids of

(a) the sequence from amino acid 75 to amino acid 102 of SEQ ID NO: 2, or

(b) a sequence having at least 65%, at least 70%, at least 75%, at least 85%, at least 95% or at least 98% amino acid sequence identity to (a),

wherein said peptide is capable of specifically binding a p11 peptide and is less than 1000 amino acids in length. Said peptide may be for example less than 500 amino acids, less than 300 amino acids, less than 200 amino acids, less than 100 amino acids or less than 50 amino acids in length.

In one aspect, a VGSC of the invention has an amino acid sequence comprising:

(a) the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4;

(b) a species or allelic variant of (a);

(c) a variant of (a) having at least 65% amino acid sequence identity thereto; or

(d) a fragment of any of (a) to (c).

Such a VGSC will retain the ability to bind a p11 protein. Such a VGSC may also retain the ability to mediate a $Na^+$ current across a membrane, such as the plasma membrane of the cell.

A suitable variant sodium channel may be derived as described below.

The present invention also relates to the discovery that the VGSC Nav 1.8 interacts with p11 protein. According to the present invention, a suitable p11 for use in the present invention may be a naturally occurring p11 peptide, or may be an artificially constructed p11 peptide. A suitable p11 peptide may be a full-length p11 protein or a species or allelic variant thereof. For example, a suitable p11 peptide may have the rat amino acid sequence given in SEQ ID NO: 4 or the human amino acid sequence given in SEQ ID NO: 8. A suitable p11 may alternatively be a species or allelic variant of the p11 peptide of SEQ ID NO: 4 or SEQ ID NO: 8.

There is no requirement that the proteins (or nucleic acids) employed in the present invention have to include the full-length "authentic" sequence of the proteins as they occurs in nature. Variants may be used (e.g. which are derived from p11 for example) which retain its activity to modify the VGSC functional expression. Modified p11 sequences according to the present invention may have a sequence at least 70% identical to the sequence of an endogenous p11 such as the rat p11 of SEQ ID NO: 4 or the human p11 of SEQ ID NO: 8. Typically there would be 75% or more, 85% or more 95% or more or 98% or more identity between the modified sequence and the authentic sequence. A variant may comprise a fragment of a naturally occurring p11 sequence. For example, a variant p11 peptide may comprise amino acids 33 to 77 of SEQ ID NO: 4 which are shown below to be involved in the modification of VGSC functional expression. Also envisaged are variant p11 peptides comprising variants, for example allelic or species variants of such fragments.

A suitable variant p11 may be a fragment of a wild type p11 or of a variant thereof as

described above. A suitable fragment may be a truncated p11, wherein, for example, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 50% or more of the original p11 sequence has been removed. A suitable fragment may consist of or comprise a fragment of a full length p11, for example, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 50% or more of a full length sequence. A suitable fragment may be any fragment which retains the ability to bind a VGSC. A fragment may be, for example, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70 80, 90 or more amino acids in length.

A suitable p11 may comprise a fragment of a wild-type or variant p11 sequence as part of its amino acid sequence. Such a variant will retain the ability to bind VGSC. A p11 fragment which retains the ability to bind VGSC may consist of or comprise the sequence of amino acids 33 to 77 of SEQ ID NO: 4. Such a p11 fragment may be, for example, 44 to 50, 44 to 60, 44 to 70, 44 to 80 amino acids in length or larger. A suitable p11 fragment may comprise a part of the sequence of amino acids 33 to 77 of SEQ ID NO: 4, for example, 5, 10, 15, 20, 25, 20, 40 or more amino acids from this region which retain the ability to bind VGSC.

Thus, in one aspect there is provided a peptide comprising at least 10, at least 15, at least 20, at least 25, at least 35 or at least 45 contiguous amino acids of
(a) the sequence from amino acid 33 to amino acid 77 of SEQ ID NO: 4, or
(b) a sequence having at least 70%, at least 75%, at least 85%, at least 95% or at least 98% amino acid sequence identity to (a),
wherein said peptide is capable of specifically binding a voltage gated sodium channel and is less than 80 amino acids in length. Said peptide may be for example less than 70 amino acids, less than 60 amino acids, less than 50 amino acids, less than 40 amino acids or less than 30 amino acids in length.

A p11 peptide for use in the methods of the present invention may therefore have an amino acid sequence comprising:

(a) the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 8;

(b) a species or allelic variant of (a);

(c) a variant of (a) having at least 70% amino acid sequence identity thereto; or

(d) a fragment of any of (a) to (c).

Such a p11 peptide will retain the ability to bind a VGSC.

The term "derived" includes variants produced by modification of the authentic native sequence e.g. by introducing changes into the full-length or part-length sequence, for example substitutions, insertions, and/or deletions. This may be achieved by any appropriate technique, including restriction of the sequence with an endonuclease followed by the insertion of a selected base sequence (using linkers if required) and ligation. Also possible is PCR-mediated mutagenesis using mutant primers. It may, for instance, be preferable to add in or remove restriction sites in order to facilitate further cloning. There may be up to five, for example up to ten or up to twenty or more nucleotide deletions, insertions and/or substitutions made to the full-length or part length sequence provided functionality is not totally lost.

Similarity or identity may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, or BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are preferably set, using the default matrix, as follows: Gapopen (penalty for the first residue in a gap): -16 for DNA; Gapext (penalty for additional residues in a gap): -4 for DNA KTUP word length: 6 for DNA. Alternatively, homology in this context can be judged by probing under appropriate stringency conditions. One common formula for calculating the stringency conditions required to achieve hybridization between (complementary) nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989): $T_m = 81.5°C + 16.6\text{Log}\,[Na+] + 0.41\,(\%\ G+C) - 0.63\,(\%\ \text{formamide}) - 600/\#bp$ in duplex. Preferred conditions will give hybridisation of molecules at least 70% homology as described above.

*Nucleic acids*

The present invention also encompasses the use of nucleic acids which encode VGSCs or p11 peptides of the invention to produce such proteins. For example, provided in the sequence listing are nucleic acid sequences encoding the rat Nav 1.8 channel (SEQ ID NO: 1), the rat p11 protein (SEQ ID NO: 3), the human Nav 1.8 channel (SEQ ID NO: 5) and the human p11 protein (SEQ ID NO: 7).

Generally, nucleic acids of, or for use in, the present invention (e.g. encoding p11) may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities. Nucleic acid according to the present invention may be in the form of, or derived from, cDNA, RNA, genomic DNA and modified nucleic acids or nucleic acid analogs.

Thus the invention also relates, in a further aspect, to use of a heterologous nucleic acid molecule which comprises a nucleotide sequence encoding p11 described above, in the various methods of the invention.

The term "heterologous" is used broadly herein to indicate that the gene/sequence of nucleotides in question (e.g. encoding p11) have been introduced into cells using genetic engineering, i.e. by human intervention. A heterologous gene may replace an endogenous equivalent gene, i.e. one which normally performs the same or a similar function, or the inserted sequence may be additional to the endogenous gene or other sequence. Nucleic acid heterologous to a cell may be non-naturally occurring in cells of that type, variety or species.

Nucleic acid sequences which encode a polypeptide in accordance with the present invention can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual", Cold

Spring Harbor Laboratory Press, 1989, and Ausubel *et al.*, Short Protocols in Molecular Biology, John Wiley and Sons, 1992). These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of the relevant nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparation of cDNA sequences.

*Constructs and Vectors*

In cell-based assay embodiments of the present invention, the polypeptide of interest can be introduced by causing or allowing the expression in a cell of an expression construct or vector.

A construct for delivery of a nucleic acid of the invention may include any other regulatory sequences or structural elements as would commonly be included in such a system, and as is described below. The vector components will usually include, but are not limited to, one or more of an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan. Nucleic acid sequences which enable a vector to replicate in one or more selected host cells are well known for a variety of bacteria, yeast, and viruses. For example, various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Particularly preferred for use herein is an expression vector e.g. in the form of a plasmid, cosmid, viral particle, phage, or any other suitable vector or construct which can be taken up by a cell and used to express a coding sequence. Expression vectors usually contain a promoter which is operably linked to the protein-encoding nucleic acid sequence of interest, so as to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter

is "under transcriptional control" of the promoter. Transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Where a cell line is used in which both the VGSC and p11 are heterologous, these proteins may be expressed from a single vector or from two separate vectors. More than one copy of the protein encoding sequences may be present in the vector.

Expression vectors of the invention may also contain one or more selection genes. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli.* The protein encoding sequences may include reporter genes which may be any suitable reporter gene used in the art. Such reporter genes includes chloramphenicol acetyl transferase (CAT), β-galactosidase, luciferase or GFP.

*Cells*

The methods referred to above may therefore further include introducing the nucleic acid into a host cell. The introduction, which may be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For example, the calcium phosphate precipitation method of Graham and van der Eb, Virology 52:456-457 (1978) can be employed. General aspects of mammalian cell host system

transformations have been described in U.S. Patent No. 4,399,216. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527 537 (1990) and Mansour et al., Nature 336:348-352 (1988).

The cells used in methods of the present invention may be present in, or extracted from, organisms, may be cells or cell lines transiently or permanently transfected or transformed with the appropriate proteins or nucleic acids encoding them or may be cells or cell lines which express the required VGSC and p11 peptide from endogenous (i.e. not artificially introduced) genes. The term "*in vivo*" where used herein includes all these possibilities. Thus *in vivo* methods may be performed in a suitably responsive cell line which expresses the VGSC (either as a native channel, or from a vector introduced into the cell). The cell line may be in tissue culture or may be, for example, a cell line xenograft in a non-human animal subject.

The cell lines used in assays of the invention may be used to achieve transient expression of p11 or may be stably transfected with constructs which express a p11 peptide. The cell lines may be transiently or stably transfected with constructs which express the VGSC. Means to generate stably transformed cell lines are well known in the art and such means may be used here. Preferred cells are non-neuronal e.g. CHO cell cells.

The level of p11 or VGSC expression in a cell may be increased by introducing it into the cells directly or by causing or allowing expression from heterologous nucleic acid encoding therefore. A cell may be used which endogenously expresses p11 and/or VGSC without the introduction of heterologous genes. Such a cell may endogenously express sufficient levels of p11 and/or VGSC for use in the methods of the invention, or may express only low levels of p11 and/or VGSC which require supplementation as described herein. A cell may be used which endogenously expresses no p11 or VGSC, but which can be made to express p11 and VGSC using methods such as those described herein.

The present invention therefore encompasses host cells which express VGSC and p11 peptide according to the present invention, one or both of which may be heterologously expressed. In such cells, said VGSC and said p11 peptide should be expressed such that the two proteins interact to upregulate the functional expression of the VGSC. Such host cells are suitable for use in the screening methods of the invention.

Host cells transfected or transformed with expression or cloning vectors described herein may be cultured in conventional nutrient media. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in "Mammalian Cell Biotechnology: a Practical Approach", M. Butler, ed. JRL Press, (1991) and Sambrook *et al, supra.*

*Transgenic organisms*

Host cells according to the present invention (i.e. including heterologous p11 for increasing VGSC expression) may be comprised in a transgenic animal, and the present invention further provides uses of the transgenic animal in the methods herein. The transgenic organisms of the invention all include within a plurality of their cells a cloned recombinant or synthetic DNA sequence which encodes the heterologous p11.

For more details regarding the production of transgenic organisms, and specifically transgenic mice, refer to U.S. Pat. No. 4,873,191, issued Oct. 10, 1989 (incorporated herein by reference to disclose methods for producing transgenic mice), and to the numerous scientific publications referred to and cited therein.

The foregoing discussion has been generally concerned with uses of the nucleic acids of the present invention for production of functional polypeptides, thereby increasing the concentration of p11 in a cell so as to increase functional expression of the

VGSC. However, as explained below, the information disclosed herein may also be used to reduce the activity of p11 in cells in which it is desired to do so, with a corresponding reduction in the functional expression of the VGSC.

*Increasing functional VGSC expression*

The present invention provides a method for enhancing the functional expression of a VGSC comprising exposing said channel to a p11 peptide. Thus the invention provides a method of modifying the translocation of a voltage gated sodium channel into a plasma membrane of a cell, which method comprises the step of altering the concentration of p11 in the cell.

Such a method may be used to increase the functional expression of a VGSC in the cell. The level of "functional expression" of the channel is used herein to describe the quantity or proportion of the channel which is active within a cell. "Active" in this context means capable of mediating a sodium current across a membrane in response to an appropriate stimulus.

Thus a further aspect of the present invention provides a method of enhancing the functional expression of a VGSC in a cell, which method comprises the step of increasing the level of p11 in the cell.

The VGSC may be any VGSC of the invention as described above. The p11 peptide may be any p11 of the invention as described above. The cell may be any suitable cell line as described above. Preferably the VGSC is expressed within the cell. The p11 peptide may also be expressed within the cell or may be applied to the cell. The VGSC and/or the p11 peptide may be expressed from endogenous genes within the cell or from heterologous genes that have been introduced into the cell, for example by transfaction of the cell with one or more vectors as described above.

Preferably, a p11 peptide of the invention is either applied to the cell or is heterologously expressed within the cell. The p11 peptide may be expressed under

the control of an inducible promoter so that the level of p11 expressed within the cell may be regulated. By heterologously providing p11 to the cell, the functional expression of the VGSC, that is the recruitment of the VGSC to the membrane and the subsequent activity of the VGSC, may be enhanced.

A cell in which the functional expression of a VGSC has been enhanced by such a method may be subsequently used in a screening method of the invention. Such a cell will have enhanced VGSC functional expression and will therefore be particularly sensitive to any changes in VGSC activity that a test compound may cause.

*Assays using enhanced VGSC functional expression*

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility.

One aspect of the present invention is based on the enhanced VGSC functionality which can be achieved using p11. This effect can be used to generate assays having enhanced sensitivity. Such systems (e.g. cell lines) are particularly useful for identifying compounds capable of modulating the VGSC.

"Modulating" herein includes any effect on the functional expression of a VGSC. This includes blocking or inhibiting the activity of the channel in the presence of, or in response to, an appropriate stimulator. Alternatively modulators may enhance the activity of the channel. Preferred modulators are channel blockers or inhibitors.

The screening methods described herein generally assess whether a test compound or putative modulator are capable of causing a change in an activity of a VGSC. Any

activity normally exhibited by a VGSC may be measured. For example, a suitable activity may be the ability of the VGSC to bind specifically to or to form a complex with a p11 peptide. Such a binding activity may be measured using methods known in the art, such as those described herein. A test compound which modulates this binding activity is a potential modulator of VGSC. Another activity of VGSCs which may be measured is the ability to function as a sodium channel. This may be measured using methods known in the art such as those described herein. For example, a test compound may affect the ability of a VGSC to produce a sodium current across a membrane in which the VGSC is present. Such assays may include the application of a specific stimulus, for example a stimulus which would normally result in sodium current flow.

The present aspect of the invention may take the form of any, preferably *in vivo*, assay utilising the enhanced VGSC functionality which can be achieved using p11. The term "*in vivo*" includes cell lines and the like as described above. This assay is carried out in a cell in which the functional expression of the VGSC has been enhanced by exposure to a p11 peptide. Thus the *in vivo* assays may be performed in a suitably responsive cell line which expresses a VGSC of the invention (either as a native channel, or from a vector introduced into the cell) and in which p11 is either applied to said cell or is expressed within said cell (endogenously or heterologously). Preferably, a p11 peptide of the invention is either applied to the cell or is heterologously expressed within the cell. The p11 peptide may be expressed under the control of an inducible promoter so that the level of p11 expressed within the cell may be regulated. By heterologously providing p11 to the cell, the functional expression of the VGSC, that is the recruitment of the VGSC to the membrane and the subsequent activity of the VGSC, may be enhanced. In the *in vivo* assays of the invention, it will be desirable to achieve sufficient levels of p11 to recruit VGSC to the membrane to enhance its functional expression. However, the precise format of the assays of the invention may be varied by those of skill in the art using routine skill and knowledge.

The invention therefore provides methods for increasing the functional expression of a VGSC in a cell, comprising exposing said VGSC to a p11 peptide of the invention. Cells having enhanced functional expression of VGSC are also envisaged as an aspect of the invention.

The invention further provides methods of modulating a VGSC the functional expression of which has been enhanced, which method comprises the step of contacting said channel with a putative modulator thereof.

The contacting step may be *in vivo* or *in vitro*, as described in more detail below. One suitable system for testing modulation (e.g. inhibition or blockage) of, for example, the SNS sodium channel (Nav 1.8), is the CHO-SNS employed in the Examples below. Other systems for testing modulation are disclosed e.g. in WO 97/01577. Membrane currents are conveniently measured with the whole-cell configuration of the patch clamp method, according to the procedure detailed in the Examples. Preferred voltage clamps are those in which the cell potential is stepped from the holding potential of about -90 mV to test potentials that range from about -110 mV to +60 to 80 mV. In order to isolate TTX-R sodium currents, TTX, 4-aminopyridine (AP) and $CdCl_2$ were used with tetraethyl ammonium ions (TEA), and Cs. However those skilled in the art will be aware of other such compounds and combinations of compounds which could be used analogously.

In one embodiment there is provided a method for identifying a modulator of a VGSC which method comprises the steps of:

(i) providing a cell in which the functional activity of said channel has been enhanced as described above (e.g. by increasing the concentration of p11 in the cell e.g. by causing or allowing expression from a nucleic acid encoding p11 in the cell);

(ii) contacting (directly or indirectly) the channel in the cell with the test compound,

(iii) measuring the activity (e.g. the current mediated by the channel, optionally in the presence of an activator) of the channel.

Preferably the activity before and after the contacting with the test compound will be compared, and optionally the relative activity will be correlated with the modulatory activity of the test compound. Compounds may therefore be identified that are capable of modulating the activity of a VGSC. Such compounds may have therapeutic use in the treatment or prevention of conditions associated with VGSC activity as described in more detail below.

Methods of the present invention may be employed in high throughput screens analogous to those well known in the art - see e.g. WO 00/16231 (Navicyte); WO 00/14540 (Tibotec); DE 19840545 (Jerini Biotools); WO 00/12755 (Higher Council for Scientific Research); WO 00/12705 (Pausch MH; Wess J); WO 00/11216 (Bristol-Myers Squibb); US 6027873 (Genencor Intl.); DE 19835071 (Carl Zeiss; F Hoffman-La Roche); WO 00/03805 (CombiChem); WO 00/02899 (Biocept); WO 00/02045 (Euroscreen); US 6007690 (Aclara Biosciences).

*Interaction between p11 and VGSC*

The interaction of a p11 peptide and a VGSC may be investigated, optionally using fragments of one or both proteins. The proteins or fragments may be labeled to facilitate this.

For example the proteins or fragments can be linked to a coupling partner, e.g. a label. Techniques for coupling labels to peptidyl coupling partners are well known in the art. Labels may be fluorescent marker compounds expressed as fusions e.g. GFP. In another embodiment the proteins or fragments may be radiolabeled. Radiolabeling of peptides can be achieved using various methods known in the art. For example, peptides can be labelled with a radioactive isotope through use of a chelating agent or by covalent labelling with a material capable of direct reaction with a peptide (such as iodine), as well as by direct labelling (substitution of a radioactive isotope, such as $^{14}C$ or tritium, for an atom present in the peptide) or $^{35}S$-methionine which may be incorporated into recombinantly produced proteins.

Generally, radiolabelled peptides containing tyrosine will be prepared using $I^{125}$, or by tritium exchange. See U.S. Patent No. 5,384,113, as well as numerous other patent and other publications, for general techniques available for the radiolabeling process. As used herein, the term "radiolabeled" describes a product that has been attached to a radioisotope by any of the various known methods, such as by covalent labeling or covalent binding, by a direct substitution method, or by a chelation method.

Other suitable detectable labels include tags such as an HA tag, GST or histidine. Recombinantly produced protein may also be expressed as a fusion protein containing an epitope which can be labelled with an antibody. Alternatively, an antibody against the proteins can be obtained using conventional methodology.

In a further aspect of the invention, the labeling methods described above are used to identify the p11 binding site on the VGSC (and vice versa). Such methods will generally comprise the steps of producing a fragment of one or both proteins, and contacting said fragment with its binding partner (all or part of it) and determining whether binding occurs. Preferably one or both partners will be labelled and\or tagged to facilitate the detection of binding.

For example, in order to identify the binding site for p11 in the VGSC, small segments of the VGSC believed to contain said binding site may be tested.

Preferred fragments may be selected from the N-terminal intracellular domain of the VGSC, for example from amino acids 1 to 127 of the rat Nav1.8 sequence as given in SEQ ID NO: 2. Preferably fragments represent sequences which are believed to be either unique to the channel, or are at least well conserved among VGSCs. Preferred fragments of the Nav 1.8 channel of SEQ ID NO: 2 may include amino acid positions 1 to 25, 26 to 50 and 51 to 127.

As described in the Examples below, it appears that the binding site for p11 on the rat Nav1.8 channel lies in the N-terminal intracellular domain between amino acids 53 and 127 (with reference to SEQ ID NO: 2), and preferably between amino acids 75 and 102. Similarly, the rat p11 protein binds to the rat Nav1.8 channel via a region at amino acids 33 to 77 (with reference to SEQ ID NO: 4). Similar methods may be used to localise or identify binding sites in other VGSCs or p11 peptides.

Binding fragments can be identified using the GST "pull down assay". This is described in more detail in the Examples hereinafter, wherein rat p11 protein produced in COS-7 cells by lipofection was mixed with fragments of SNS (Nav1.8) N-terminal which were fused to GST made in bacteria. These protein complexes are collected by glutathione beads and p11 is recovered only when the VGSC fragment has one or more binding site(s) for it. In other embodiments, co-immunoprecipitation or an overlay assay can be done in place or in addition to the "pull down" assay.

After narrowing down to one (or more) of the N-terminal fragments, the binding site can be further investigated e.g. using point mutations by recombinant PCR or a uracil containing vector system (Journal of Physiology (1999) 516.2, 433-446 cAMP-dependent phosphorylation of the tetrodotoxin-resistant voltage-dependent sodium channel SNS. E.M. Fitzgerald, K. Okuse, J.N. Wood, A.C. Dolphin, S.J. Moss). Since the target cDNA (e.g. corresponding to a fragment described above of about one third of N-terminal domain) may be fairly short, recombinant PCR may be preferred. Mutated N-terminal fragments may again be tested e.g. in the GST "pull down" assay, to precisely identify the interaction site between the VGSC and p11.

Once identified the binding site may be modeled in 3 dimensions to produce mimetics. Alternatively it may be used directly e.g. as a binding partner (optionally in phage display) to screen for compounds.

*Assay for modulators of interaction*

In a further aspect the present invention provides an assay for a modulator of the functional expression of VGSC in a cell, which assay comprises the steps of:

a) bringing into contact a VGSC, a p11 peptide, and a putative modulator compound under conditions where the VGSC and the p11, in the absence of modulator, are capable of forming a complex; and

b) measuring the degree of inhibition of complex formation caused by said modulator compound.

The present invention further provides an assay for a modulator of the functional expression of a VGSC in a cell, which assay comprises the steps of:

a) bringing into contact a VGSC, a p11 peptide, and a putative modulator compound under conditions where the VGSC and the p11, in the absence of modulator, are capable of forming a complex; and

b) exposing the VGSC to a stimulus such as to produce to a sodium current across a membrane in which the VGSC is present,

c) measuring the degree of inhibition of the current caused by said modulator compound.

An inhibition in the current indicates that the compound is a potential modulator of VGSC activity. Such a compound may have therapeutic use in the treatment or prevention of conditions associated with VGSC activity, as described in more detail below.

One assay format which is widely used in the art to study the interaction of two proteins is a two-hybrid assay. This assay may be adapted for use in the present invention. A two-hybrid assay comprises the expression in a host cell of the two proteins, one being a fusion protein comprising a DNA binding domain (DBD), such as the yeast GAL4 binding domain, and the other being a fusion protein comprising an activation domain, such as that from GAL4 or VP16. In such a case the host cell (which may be bacterial, yeast, insect or mammalian, particularly yeast or mammalian) will carry a reporter gene construct with a promoter comprising a DNA

binding elements compatible with the DBD. The reporter gene may be a reporter gene such as chloramphenical acetyl transferase, luciferase, green fluorescent protein (GFP) and β-galactosidase, with luciferase being particularly preferred.

Two-hybrid assays may be in accordance with those disclosed by Fields and Song, 1989, Nature 340; 245-246. In such an assay the DNA binding domain (DBD) and the transcriptional activation domain (TAD) of the yeast GAL4 transcription factor are fused to the first and second molecules respectively whose interaction is to be investigated. A functional GAL4 transcription factor is restored only when two molecules of interest interact. Thus, interaction of the molecules may be measured by the use of a reporter gene operably linked to a GAL4 DNA binding site which is capable of activating transcription of said reporter gene.

Thus two hybrid assays may be performed in the presence of a potential modulator compound and the effect of the modulator will be reflected in the change in transcription level of the reporter gene construct compared to the transcription level in the absence of a modulator.

Host cells in which the two-hybrid assay may be conducted include mammalian, insect and yeast cells, with yeast cells (such as *S. cerivisiae* and *S. pombe*) being particularly preferred.

The interaction between p11 and a VGSC may also be assessed in mammalian cells. Cells or cell lines are derived which (over) express the VGSC in a zero p11 background or in the background of endogenously expressed p11 or in the background of (over)expressed p11. This can be done by (co)transfecting the VGSC with or without p11 into the cell. Any cell may be chosen and VGSC expression and/or p11 expression may be transient or stable. The effect of p11 on the VGSC can be determined by comparing ion flux across the channel in cells (over)expressing p11 with those that do not (over)express p11 or show low levels of p11 expression. Other ways of measuring the effect of p11 on the VGSC are by assaying the extent of

membrane localisation of the VGSC in whole cells or in isolated membranes. VGSC localisation can be assessed by antibody staining in cellular immunofluorescence assays, or by western blotting of membrane fractions or by toxin binding on whole cells or membrane fractions. The interaction can also be derived in co-immunoprecipitation assays of p11 and VGSC. Inhibitors of the interaction will inhibit the functionality or the membrane localisation of VGSC, or the extent of co-immunoprecipitation between p11 and VGSC in the cells (over)expressing p11.

Another assay format measures directly, *in vivo* or *in vitro,* the interaction between p11 and the VGSC by labelling one of these proteins with a detectable label, as described above, and bringing it into contact with the other protein which has been optionally immobilised on a solid support, either prior to or after proteins have been brought into contact with each other.

The protein which is optionally immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known *per se*. In the Examples which follow a preferred *in vitro* interaction is illustrated which utilises a fusion protein of the SNS sodium channel (Nav1.8) fused to glutathione-S-transferase (GST). Such a fusion protein may be immobilized on glutathione sepharose or agarose beads.

In an *in vitro* assay format of the type described above the putative inhibitor compound can be assayed by determining its ability to diminish the amount of labelled p11 (e.g. the GFP-fusion described hereinafter) which binds to the immobilized (e.g. GST-SNS) sodium channel. This may be determined by fractionating the glutathione beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

Another assay format is dissociation enhanced lanthanide fluorescent immunoassay

(DELFIA) (Ogata *et al*, 1992). This is a solid phase based system for measuring the interaction of two macromolecules. Typically one molecule (either VGSC or p11) is immobilised to the surface of a multi well plate and the other molecule is added in solution to this. Detection of the bound partner is achieved by using a label consisting of a chelate of a rare earth metal. This label can be directly attached to the interacting molecule or may be introduced to the complex via an antibody to the molecule or to the molecules epitope tag. Alternatively, the molecule may be attached to biotin and a streptavidin-rare earth chelate used as the label. The rare earth used in the label may be europium, samarium, terbium or dysprosium. After washing to remove unbound label, a detergent containing low pH buffer is added to dissociate the rare earth metal from the chelate. The highly fluorescent metal ions are then quantitated by time resolved fluorimetry. A number of labelled reagents are commercially available for this technique, including streptavidin, antibodies against glutathione-S-transferase and against hexahistidine.

In an alternative mode, the one of the two proteins may be labelled with a fluorescent donor moiety and the other labelled with an acceptor which is capable of reducing the emission from the donor. This allows an assay according to the invention to be conducted by fluorescence resonance energy transfer (FRET). In this mode, the fluorescence signal of the donor will be altered when the two proteins interact. The presence of a candidate modulator compound which modulates the interaction will increase or decrease the amount of unaltered fluorescence signal of the donor.

FRET is a technique known per se in the art and thus the precise donor and acceptor molecules and the means by which they are linked to the p11 and a VGSC protein may be accomplished by reference to the literature.

The interaction between a VGSC and p11 may also be measured by fluorescence polarisation. Typically, binding partners are obtained as isolated peptides through chemical synthesis or as recombinant peptides or as purified peptides from tissue or cell sources. Full length p11 or fragments thereof may be employed in combination

with VGSC peptides representing, for example, the full N-terminal cytoplasmic portion or parts thereof. For example, in the case of the rat Nav1.8 channel, a suitable fragment may comprise amino acids 53 to 127 or 75 to 102 of SEQ ID NO: 2. In the case of the rat p11 protein, suitable fragments may comprise amino acids 33 to 77 of SEQ ID NO: 4.

Either of the two peptides in the assay is labelled with a suitable label, typically a fluorescent label. The fluorescent peptide is placed in a sample tube and monochromatic light is passed through a polarizing filter onto the sample tube. The fluorophore will be excited by the polarised light bundle and the emitted light is measured. The emitted light will be scattered in all directions, because of the rotational behaviour of the small peptide in solution. This rotational behaviour changes when the peptide interacts with its larger binding partner, resulting in retention of the polarisation and reduced scatter of the emitted light. Inhibitors will be screened by reading out the changes in rotational energy of the complex from the degree of polarisation of the emitted light.

Suitable fluorescent donor moieties are those capable of transferring fluorogenic energy to another fluorogenic molecule or part of a compound and include, but are not limited to, coumarins and related dyes such as fluoresceins, rhodols and rhodamines, resorufins, cyanine dyes, bimanes, acridines, isoindoles, dansyl dyes, aminophthalic hydrazines such as luminol and isoluminol derivatives, aminophthalimides, aminonaphthalimides, aminobenzofurans, aminoquinolines, dicyanohydroquinones, and europium and terbium complexes and related compounds.

Suitable acceptors include, but are not limited to, coumarins and related fluorophores, xanthenes such as fluoresceins, rhodols and rhodamines, resorufins, cyanines, difluoroboradiazaindacenes, and phthalocyanines.

A preferred donor is fluorescein and preferred acceptors include rhodamine and

carbocyanine. The isothiocyanate derivatives of these fluorescein and rhodamine, available from Aldrich Chemical Company Ltd, Gillingham, Dorset, UK, may be used to label the p11 and ER. For attachment of carbocyanine, see for example Guo et al, J. Biol. Chem., 270; 27562-8, 1995.

Rather than using fluorescence detection, it may be preferred in assay formats to detect labels and interactions using surface enhanced Raman spectroscopy (SERS), or surface enhanced resonance Raman spectroscopy (SERRS) (see e.g. WO97/05280).

An alternative assay format is a Scintillation proximity assay (SPA, Amersham Biosciences, UK). SPA uses microscopic beads containing scintillant that can be stimulated to emit light. This stimulation event only occurs when radiolabelled molecules of interest are bound to the surface of the bead. Specific bead types may be produced with different coatings for specific applications including; receptor-ligand binding, enzyme assays, radioimmunoassays, protein-protein and protein-DNA interactions.

*Modulators of interaction*

For the screening methods of the invention, any compounds may be used which may have an effect on VGSC functional expression. Such an effect may, for example, be mediated by a direct effect on the channel, or indirectly by blocking or preventing the interaction between p11 and the VGSC.

In one aspect, a compound for use in downregulating functional expression of a VGSC may be a compound which binds specifically to the VGSC and/or the p11 peptide. For example, such a compound may bind to the intracellular domain of the VGSC, such as in the region of amino acids 53 to 127 or 75 to 102 of the rat Nav 1.8 sodium channel as given in SEQ ID NO: 2 , or an equivalent region of a variant channel, or may bind in the region of amino acids 33 to 77 of a p11 peptide as given in SEQ ID NO: 4, or an equivalent region of a variant p11 peptide. A compound

may therefore prevent binding between the VGSC and the p11 peptide and thereby prevent the enhancement of VGSC functional expression normally caused by p11.

Compounds (putative VGSC modulators) which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used. In preferred embodiments the substances may be provided e.g. as the product of a combinatorial library such as are now well known in the art (see e.g. Newton (1997) Expert Opinion Therapeutic Patents, 7(10): 1183-1194). The amount of putative modulator compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 to 100 nM concentrations of putative modulator compound may be used, for example from 0.1 to 10 nM. Modulator compounds may be those which either agonise or antagonise the interaction. Antagonists (inhibitors) of the interaction are particularly desirable.

In a further aspect, the present invention provides peptide compounds, and processes for devising and producing such compounds, which are based on the portions of the VGSC and p11 which interact with each other e.g. the regions described in the Examples below.

Modulators which are putative inhibitor compounds can be derived from the p11 and VGSC protein sequences. Peptide fragments of from 5 to 40 amino acids, for example from 6 to 10 amino acids from the region of p11 and VGSC which are responsible for the interaction between these proteins may be tested for their ability to disrupt this interaction. For example, such peptides may be derived from the intracellular domain of the VGSC such as the region of amino acids 53 to 127 or 75 to 102 of the rat Nav1.8 sodium channel as given in SEQ ID NO: 2, or from amino acids 33 to 77 of the rat p11 protein as given in SEQ ID NO: 4.

Antibodies directed to the site of interaction in either protein form a further class of

putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction between p11 and VGSC. A suitable antibody may bind to either the VGSC or the p11 peptide, and thereby prevent or block the interaction between these molecules.

Antibodies may be raised against specific epitopes of the VGSC or p11 peptide of the invention. For example, antibodies may be raised specifically against those regions, as described above, which are involved in the interaction between the VGSC and the p11 peptide.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments which bind a VGSC or p11 peptide of the invention. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies. Furthermore, the antibodies and fragment thereof may be chimeric antibodies, CDR-grafted antibodies or humanised antibodies.

Antibodies of the invention can be produced by any suitable method. Means for preparing and characterising antibodies are well known in the art, see for example Harlow and Lane (1988) "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. For example, an antibody may be produced by raising antibody in a host animal against the whole polypeptide or a fragment thereof, for example an antigenic epitope thereof, herein after the "immunogen".

A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the animal's serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified.

A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein (1975) *Nature* **256**, 495-497).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

An antibody, or other compound, "specifically binds" to a protein when it binds with preferential or high affinity to the protein for which it is specific but does substantially bind not bind or binds with only low affinity to other proteins. A variety of protocols for competitive binding or immunoradiometric assays to determine the specific binding capability of an antibody are well known in the art (see for example Maddox *et al*, J. Exp. Med. 158, 1211-1226, 1993). Such immunoassays typically involve the formation of complexes between the specific protein and its antibody and the measurement of complex formation.

In a further aspect, decreased functional expression of a VGSC may be achieved by inhibiting the expression from the VGSC gene. For example, down-regulation of expression of a target gene may be achieved using anti-sense technology or RNA

interference.

In using anti-sense genes or partial gene sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. See, for example, Smith *et al*,(1988) *Nature* 334, 724-726. Such methods would use a nucleotide sequence which is complementary to the coding sequence. Further options for down regulation of gene expression include the use of ribozymes, e.g. hammerhead ribozymes, which can catalyse the site-specific cleavage of RNA, such as mRNA (see e.g. Jaeger (1997) The new world of ribozymes Curr Opin Struct Biol 7:324-335, or Gibson & Shillitoe (1997) Ribozymes: their functions and strategies form their use Mol Biotechnol 7: 242-251.)

RNA interference is based on the use of small double stranded RNA (dsRNA) duplexes known as small interfering or silencing RNAs (siRNAs). Such molecules are capable of inhibiting the expression of a target gene that they share sequence identity or homology to. Typically, the dsRNA may be introduced into cells by techniques such as microinjection or transfection. Methods of RNA interference are described in, for example, Hannon (2002) RNA Interference, Nature 418: 244-251 and Elbashir et al (2001) Duplexes of 21-nucleotide RNAs mediate RNA interferenec in cultured mammalian cells, Nature 411: 494-498.

*Specificity of modulation*

Where any of the methods of identifying modulators of the VGSC utilizes a cell-based system, such methods may further include the step of testing the viability of the cells in the assay e.g. by use of a lactate dehydrogenase assay kit (Sigma). This step may provide an indication of any interference by the test agent of vital cellular functions.

*Therapeutic compositions and their use*

As used hereafter the term "VGSC modulator" is intended to encompass any and all of the above modulator compounds which may be identified using any of the assays or design methods of the invention. Such VGSC modulators identified by the methods of the present invention may be isolated, purified, formulated in a composition, such as a pharmaceutical composition, and/or used in therapy as described below.

VGSC modulators as described above may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other materials from their source or origin. Where used herein, the term "isolated" encompasses all of these possibilities. They may optionally be labeled or conjugated to other compounds.

VGSC modulators may be useful in the treatment or prophylaxis of a wide range of disorders. Thus in a further aspect, the present invention provides a pharmaceutical composition comprising a VGSC modulator peptide or encoding nucleic acid molecule and its use in methods of therapy or diagnosis.

In a further aspect, the present invention provides a pharmaceutical composition comprising one or more VGSC modulators as defined above and its use in methods of therapy or diagnosis.

In further aspects, the present invention provides the above VGSC modulators and nucleic acid molecules for use in the preparation of medicaments for therapy.

In one aspect, the invention includes a method of producing analgesia in a mammalian subject, which method includes administering to the subject a VGSC modulator of the present invention. Modulators of the channel may prevent transmission of impulses along sensory neurons and thereby be useful in the treatment or prevention of acute, chronic or neuropathic pain.

Acute pain is temporary, generally lasting a few seconds or longer. Acute pain usually starts suddenly and is generally a signal of rapid-onset injury to the body or intense smooth muscle activity. Acute pain can rapidly evolve into chronic pain. Chronic pain generally occurs over a longer time period such as weeks, months or years.

The VGSC modulators of the invention may be used in the treatment or prevention of acute or chronic pain, or to prevent acute pain evolving into chronic pain. Treatment of pain is intended to include any level of relief from the symptoms of pain, from a decrease in the level of pain to complete loss of the pain. Prevention includes the prevention of the onset of pain, and the prevention of the worsening of pain, for example the worsening of pain symptoms or the progression from acute pain to chronic pain.

Examples of types of chronic pain which may be treated or prevented with the VGSC modulators of the present invention include osteoarthritis, rheumatoid arthritis, neuropathic pain, cancer pain, trigeminal neuralgia, primary and secondary hyperalgesia, inflammatory pain, nociceptive pain, tabes dorsalis, phantom limb pain, spinal cord injury pain, central pain, post-herpetic pain and HIV pain, noncardiac chest pain, irritable bowel syndrome and pain associated with bowel disorders.

In a further aspect there is provided a method of preventing progression of pain in a subject at risk for developing such pain, comprising administering to the subject a VGSC modulator of the present invention.

A composition may be administered alone or in combination with other treatments (e.g. treatments having analgesic effect such as NSAIDS), either simultaneously, separately or sequentially, dependent upon the condition to be treated.

The VGSC modulators can be formulated into pharmaceutical compositions. These compositions may comprise, in addition to one of the above substances, a

pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

For delayed release, the modulators may be included in a pharmaceutical composition for formulated for slow release, such as in microcapsules formed from biocompatible polymers or in liposomal carrier systems according to methods known in the art.

For continuous release of peptides, the peptide may be covalently conjugated to a water soluble polymer, such as a polylactide or biodegradable hydrogel derived from an amphipathic block copolymer, as described in U.S. Pat. No. 5,320,840. Collagen-based matrix implants, such as described in U.S. Pat. No. 5,024,841, are also useful for sustained delivery of peptide therapeutics. Also useful, particularly for subdermal

slow-release delivery to perineural regions, is a composition that includes a biodegradable polymer that is self-curing and that forms an implant in situ, after delivery in liquid form. Such a composition is described, for example in U.S. Pat. No. 5,278,202.

Peptides (for example such as those designed or discovered to inhibit the interaction of p11 and a VGSC as described above) may preferably be administered by transdermal iontophoresis. One particularly useful means for delivering compound to perineural sites is transdermal delivery. This form of delivery can be effected according to methods known in the art. Generally, transdermal delivery involves the use of a transdermal "patch" which allows for slow delivery of compound to a selected skin region. Although such patches are generally used to provide systemic delivery of compound, in the context of the present invention, such site-directed delivery can be expected to provide increased concentration of compound in selected regions of neurite proliferation. Examples of transdermal patch delivery systems are provided by U.S. Pat. No. 4,655,766 (fluid-imbibing osmotically driven system), and U.S. Pat. No. 5,004,610 (rate controlled transdermal delivery system).

For transdermal delivery of peptides transdermal delivery may preferably be carried out using iontophoretic methods, such as described in U.S. Pat. No. 5,032,109 (electrolytic transdermal delivery system), and in U.S. Pat. No. 5,314,502 (electrically powered iontophoretic delivery device).

For transdermal delivery, it may be desirable to include permeation enhancing substances, such as fat soluble substances (e.g., aliphatic carboxylic acids, aliphatic alcohols), or water soluble substances (e.g., alkane polyols such as ethylene glycol, 1,3-propanediol, glycerol, propylene glycol, and the like). In addition, as described in U.S. Pat. No. 5,362,497, a "super water-absorbent resin" may be added to transdermal formulations to further enhance transdermal delivery. Examples of such resins include, but are not limited to, polyacrylates, saponified vinyl acetate-acrylic acid ester copolymers, cross-linked polyvinyl alcohol-maleic anhydride copolymers,

saponified polyacrylonitrile graft polymers, starch acrylic acid graft polymers, and the like. Such formulations may be provided as occluded dressings to the region of interest, or may be provided in one or more of the transdermal patch configurations described above.

In yet another embodiment, the compound is administered by epidural injection. Membrane permeation enhancing means can include, for example, liposomal encapsulation of the peptide, addition of a surfactant to the composition, or addition of an ion-pairing agent. Also encompassed by the invention is a membrane permeability enhancing means that includes administering to the subject a hypertonic dosing solution effective to disrupt meningeal barriers.

The modulators can also be administered by slow infusion. This method is particularly useful, when administration is via the intrathecal or epidural routes mentioned above. Known in the art are a number of implantable or body-mountable pumps useful in delivering compound at a regulated rate. One such pump described in U.S. Pat. No. 4,619,652 is a body-mountable pump that can be used to deliver compound at a tonic flow rate or at periodic pulses. An injection site directly beneath the pump is provided to deliver compound to the area of need, for example, to the perineural region.

In other treatment methods, the modulators may be given orally or by nasal insufflation, according to methods known in the art. For administration of peptides, it may be desirable to incorporate such peptides into microcapsules suitable for oral or nasal delivery, according to methods known in the art.

Whether it is a peptide, antibody, nucleic acid molecule, small molecule or other pharmaceutically-useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the

individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

Instead of administering these agents directly, they could be produced in the target cells by expression from an encoding gene introduced into the cells, e.g. in a viral vector (a variant of the VDEPT technique- see below). The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are switched on more or less selectively by the target cells.

Alternatively, the agent could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT; the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO90/07936).

The expression of p11 in an organism may be correlated with the functional expression of a VGSC in the organism, and this correlation may form the basis of diagnosis of diseases related to inappropriate VGSC expression.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these. Any reference mentioned herein, inasmuch as it may be required to supplement the common general knowledge of the

person skilled in the art in practicing the invention, is specifically incorporated herein by reference in its entirety.

**Examples**

Materials and Methods

*Yeast two-hybrid screen*

A two-hybrid interaction screen was performed with the N-terminal intracellular domain (amino acid position 1-127) of $Na_V1.8$ and a rat P1 DRG cDNA library as described[11]. The bait plasmid was generated by PCR with rat $Na_V1.8$ cDNA as a template with a forward primer (5'-gcgaattcatggagctccctttg-3') and a reverse primer (5'-tatagcggccgctttgatggctgttcttc-3'). The amplified fragment was ligated into pEG202 at EcoRI–NotI sites as an in-frame fusion with the LexA–DNA binding domain.

A cDNA library from postnatal day 1 dorsal root ganglia (DRG) was generated[11]. The DRG library cDNAs were expressed as in-frame fusions with the Gal4 transcriptional activation domain. Approximately 5 x $10^6$ yeast transformants were screened for β-galactosidase activity and growth in the absence of leucine. and five identical positive clones encoding a full length p11 were identified. The clone included a 51 bp 5'-UTR, a 288 bp coding region, and a 450 bp 3'-UTR of the rat p11 gene. To verify that p11 interacts specifically with the N-terminal intracellular domain of $Na_V1.8$, the rescued p11-encoding plasmid DNA was re-introduced into other strains of yeast containing different intracellular domains of $Na_V1.8$ as baits. Direct interaction between p11 and N-terminal domain of $Na_V1.8$ in vitro was assessed using GST pull down assays[12]. Fuller details of experimental methods are present in supplementary information.

*GST pull-down assay*

The segment encoding the N-terminus of rat $Na_V1.8$ (amino acid position 1-127) was

amplified by PCR and cloned in-frame into the EcoRI/NotI sites of pGEX-5X-1 (Amersham Pharmacia Biotech). The GST/$Na_V1.8$ N-terminus fusion protein, designated as GST-SNS(I), was produced in E. coli BL-21 and affinity purified on glutathione-sepharose beads. Original pGEX-5X-1 was used to produce GST control protein.

Full length rat cDNA for p11 in yeast expression vector pJG4-5 was subcloned as the NcoI-XbaI fragment into the pBS500 expression vector which results in GFP-p11 fusion protein expression driven by elongation factor 2α promoter. The resultant plasmid was designated as pBS-GFP/p11. COS-7 cells were transfected with pBS-GFP/p11 by lipofection. GFP-p11 fusion protein was extracted from transfected COS-7 cells by lysis buffer (4% SDS, 10 mM sodium phosphate, pH 7.4) 3 days after transfection. The lysate was centrifuged at 10,000rpm at 4°C for 10mins. The supernatant was retained and centrifuged at 14,000rpm at 4°C for 30 min. The supernatant was then transferred to a macrosep 30K omega centrifugal concentrator (Pall Filtron) and 10ml ice-cold Phosphate Buffered Saline (PBS), pH 7.4, was added. It was centrifuged at 5,000rpm for 2 hours. The filtrate was discarded and the residue, containing the solubilized protein, was used in the pull-down assay experiment. GFP expression plasmid, pBS-GFP, was used for production of GFP protein for a negative control.

To examine the binding of p11 to the N-terminus of $Na_V1.8$ *in vitro*, glutathione-sepharose beads pre-incubated with purified GST, serving as control, or GST-SNS(I) were incubated at 4°C overnight with GFP-p11 fusion protein or GFP protein extracted from transfected COS cells in PBS. After intensive washing with ice-cold PBS, the bound proteins were denatured in sample buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% Bromophenol Blue, 20% Glycerol, 200mM DDT), separated by 10% SDS-PAGE, and transferred to nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech). The membrane was blocked for 1 h in 5% nonfat dry milk in PBS-T at room temperature (0.1% Tween-20 in PBS, pH7.4). Primary anti-GFP

antibody (Santa Cruz Biotechnology) in 1:800 dilution was applied for 1 hr at room temperature. Secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse IgG, Amersham Pharmacia Biotech) in 1:2000 dilution was applied for 1 hr at room temperature. ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech) was applied according to the manufacturer's instructions and the blot was exposed to BioMax film (Kodak).

*Northern Blot analysis*

Total RNA was isolated from various rat tissues by acid guanidinium thiocyanate-phenol-chloroform extraction method[16] and fractioned by electrophoresis on 1.5% agarose gel in 10mM phosphate buffer (pH 6.5) after glyoxylation. RNA was blotted onto hybond $N^+$ nylon membrane in 20 x SSC solution for 6 hours and fixed by UV irradiation. The membrane was stained with methylene blue to confirm even recoveries of RNA. Thereafter, the membrane was prehybridized and hybridized with $^{32}$P-labeled p11 DNA probe (50 ng, specific activity 2 x $10^9$ c.p.m. per µg DNA) at 43°C in hybridization buffer (50% formamide, 6 x SSC, 50mM $NaH_2PO_4$, 1mM EDTA, 1% SDS, 2.5 x Denhardt's solution, 250µg herring sperm DNA, pH 6.5) for 18 hrs. The $^{32}$P-labeled p11 DNA probe was generated from 284bp PCR fragment of p11 (amino acid position 3-127) using Klenow enzyme and random primer in the presence of [$\alpha$-$^{32}$P] ATP at 37°C for 15 min. Unincorporated nucleotides were removed using the QIA quick nucleotide removal kit (Qiagen). Hybridized membrane was washed with 0.2 x SSC/0.1% SDS at 53$^0$C and exposed to BioMax film (Kodak) at –70$^0$C.

*RT-PCR*

DRG neurons from 2 weeks old rats were cultured in DMEM containing 3.3 ng/ml aphidicoline for 7 days. Cultures were supplemented with NGF (50 ng/ml) or grown in the absence of NGF and in the presence of rabbit anti-NGF antiserum. Total RNA extracted from the culture was treated with DNase I and cDNA was synthesized with Superscript using randam hexamer. PCR (94 °C, 1 min; 58 °C, 1 min 30 sec; 72 °C,

1 min 30 sec; 50 µl) was started with the primer pair specific for p11 (284 bp), 5'-CATCCCAAATGGAGCATG-3', 5'-CTACTTCTTCTGCTTCATGTGTACTAC-3'. After 2 cycles, the primer pair for CGRP (222 bp), 5'-GGTGTGGTGAAGGACAAC-3', 5'-CATAGGGAGAAGGGTTTC-3' was added. In separate tubes, PCR was started with the primer pair for CGRP, and the primer pair for cyclophilin (300 bp), 5'-ACCCCACCGTGTTCTTCGAC-3', 5'-CATTTGCCATGGACAAGATG-3', were added to the reaction 3 cycles after the start. Ten µl of the PCR solution was collected every 3 cycles and applied for 1.5 % agarose gel electrophoresis and the PCR products were visualized by ethidium bromide. The intensity of each band was analyzed using NIH Image program and plotted. The linear range of amplification (p11, 32-38 cycles; CGRP, 30-36 cycles; cyclophilin 27-33 cycles) has been identified. The midpoint of this range (p11, 35 cycles; CGRP, 33 cycles; cyclophilin, 30 cycles) were used for the RT-PCR experiments and 1 µl of the reverse transcribed solutions was used for PCR.

*Immunofluorescence analysis*

A stably transformed CHO cell line (CHO-SNS22 cells) that expresses rat $Na_V1.8$ protein in the cytosol was transfected with the expression plasmid pBS-GFP/p11 by lipofection. The CHO-SNS22 cell line was kept in Nutrient Mixture F-12 (Ham) medium (GibcoBRL) with 2.5% fetal bovine serum and 1mg/ml Geneticin G418 sulphate. One day prior to transfection, cells were subcultured and plated in 35mm dish containing F-12 medium with 0.5% fetal bovine serum and 1mg/ml G418. Prior to transfection, cells in 35mm dish were rinsed twice with serum-free F-12 medium. 1.1µg of DNA was mixed with 5µl of Lipofectamine (GibcoBRL) and incubated at room temperature for 30 min. The mixture was added to the pre-rinsed cells and incubated at 37°C for 2 hours. DNA/lipofectamine mixture was replaced with F-12 medium with 0.5% fetal bovine serum and 1mg/ml G418 after 2 hours. Three days after transfection, the cells were fixed with 4% paraformaldehyde for 15 min on ice and subsequently incubated with anti-SNS polyclonal antibody (SNS11). The cells were washed with PBS and incubated with rhodamine-labelled anti-rabbit IgG before

analysis with a confocal microscope.

*In situ hybridisation*

A 284bp p11 PCR fragment was subcloned into pGEM-T Easy (Promega), and DIG-UTP labelled sense or antisense cRNA probe were generated using T7 RNA polymerase for *in situ* hybridisation studies. Frozen DRG sections (10 μm thick) were fixed for 15 min in 4% paraformaldehyde on ice and were acetylated in 0.1M triethanolamine, 0.25% acetic anhydride for 10 min. Prehybridization was carried out in 50% formamide, 4 x SSC, 100 μg/ml herring sperm DNA, 50 μg/ml tRNA, 2 x Denhardt's solution at room temperature for 1 hr. Hybridization was carried out in the same buffer containing 50 ng/ml cRNA probe at 65°C for 16 hrs. Sections were washed in 0.1 x SSC at 72°C and incubated with alkaline phosphatase conjugated anti-digoxygenin antibody (Roche). The same sections were then stained with anti-$Na_V1.8$ polyclonal antibody (SNS11) followed by rhodamine-conjugated anti-rabbit IgG antibody.

*NGF regulation of p11*

DRG neurons from 2 week old rats were cultured with NGF (50 ng/ml) or grown in the absence of NGF and in the presence of rabbit anti-NGF antiserum. Total RNA extracted from the culture was treated with DNase I and cDNA was synthesized with Superscript using randam hexamer. PCR was performed with the primer pair specific for p11 (284 bp), 5'-CATCCCAAATGGAGCATG-3', 5'-CTACTTCTTCTGCTTCATGTGTACTAC-3'.

*Transfection and extraction of p11 in COS-7 cells*

COS-7 cells were transiently transfected with 20 μg of pBS-GFP/p11 by lipofection. Three days after the transfection, the cells were incubated in the lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5) for 30 min on ice. The lysis buffer was centrifuged for 30 min at 10,000g at 4 °C. The supernatant was used for *in vitro* binding assay. GFP expression plasmid, pBS-GFP, was used

for production of GFP protein as a negative control.

*Expression of GST-SNS sodium channel fusion protein*

cDNA for $NH_2$-terminal intracellular domain of rat SNS sodium channel was amplified by PCR using primers 5'-GGAATTCATGGAGCTCCCCTTTGCG-3' and 5'-AATTGCGGCCGCAGACGCTTTGATGGCTGT-3'. The amplified fragment, corresponds to the amino acid position 1 to 127 of rat SNS sodium channel protein, was cloned into EcoRI/NotI sites in GST gene fusion vector pGEX-5X-1 . The resultant expression vector coding GST/SNS sodium channel $NH_2$-terminal fusion protein was designated as pGEX-5X-1-SNS(I). pGEX-5X-1-SNS(I) was transformed into *E. coli* strain BL21, subsequently GST/SNS(I) fusion protein was affinity purified on glutathione-Sepharose beads. The glutathione-Sepharose / GST/SNS sodium channel complex was incubated with the extract obtained from COS-7 cells transfected with pBS-GFP/p11 in binding buffer (10% glycerol, 1 mM $MgCl_2$, 100 mM KCl, 0.5 mg/ml bovine serum albumin, 10 mM Tris, pH7.9) for 4 hr at 4°C. The bound proteins were denatured in sample buffer and separated by 12% SDS-PAGE. The GFP/SNS sodium channel(I) fusion protein was detected by immunoblotting with anti-HA antibody whose epitope tag is situated in the junction between GFP and SNS sodium channel $NH_2$-terminal protein. pGEX-5X-1 was used for production of GST protein as a negative control.

*Electrophysiology*

Membrane currents were recorded from CHO-SNS 22 cells using the whole-cell patch-clamp technique. The extracellular recording solution contained the following (in mM): NaCl (140), TEA Cl (10) HEPES (10), $CaCl_2$ (2.1), $MgCl_2$ (2.12), 4-aminopyridine (4-AP) (0.5), KCl (7.5), tetrodotoxin (TTX) (250 nM). The solution was buffered to pH 7.2-3 with the addition of NaOH. The intracellular solution contained the following (in mM): CsCl (145), EGTA Na (3 (6)), Hepes (10), $CaCl_2$, (1.21), $MgCl_2$ (1.21), TEA Cl (10) and was buffered to pH 7.2-3 with the addition of CsOH. For recordings from neurons the extracellular solution was the same, except that NaCl was reduced to 43.3mM with equivalent replacement of TEA-Cl and the

addition of 20μM CdCl2. In the intracellular recording solution, 10% of the CsCl was replaced by CsF, the MgCl2 replaced by 3mM ATP (Mg) and the solution contained 500mM GTP (Li) Chemicals were either 'AnalaR' (BDH, Merk Ltd.) or supplied by Sigma. Chemicals were either 'AnalaR' (BDH, Merk Ltd., Lutterworth, Leicestershire, UK.), or supplied by Sigma (Poole, Dorset, UK). TTX was obtained from Alomone labs (TCS Biologicals, Botolph Claydon, Bucks, UK). A minority of CHO-SNS 22 cells generate an endogenous tetrodotoxin-sensitive (TTX-s) $Na^+$ current (personal observation) which was eliminated from all recordings by including 250 nM TTX in the extracellular media. No inward currents were recorded in non-transfected cells under these circumstances.

Electrodes were fabricated from thin-wall glass capillaries (GC150TF-10; Harvard apparatus, Edenbridge, Kent, UK), and had an access resistance of 2-4 MΩ when filled with recording solution. Recordings were made using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Pulse protocols were generated and data stored to disk using pClamp6 software (Axon Instruments), running on a PC. CHO-SNS 22 cells were held at –90 mV. Voltage-clamp protocols incorporated a negative pre-pulse to –110 mV, and the cell was subsequently stepped to more depolarized potentials for 50 ms (up to a final value of +80 mV), in 10 mV increments.

All experiments were performed at room temperature.

*Antisense studies*

The 309bp NcoI fragment of p11 was cloned in 3' to 5' direction into Nco-I restriction site in pBS500 vector resulted in a expression system for a sense-GFP/antisense-p11 fusion RNA, pBS-GFP/AS(p11). 400 μg/ml of pBS-GFP/AS(p11) together with 0.5% Texas Red in injection buffer (118mM KCl, 5mM Hepes, 22.2mM $NaHCO_3$, 1.2 mM $MgCl_2$, pH 7) were injected into nuclei of 2 week old rats DRG small diameter neurons using Eppendorf microinjector. During the

injection, the DMEM was replaced by a calcium free buffer (PBS and containing 10mM glucose and 2.4mM $MgCl_2$). After completion of the injections, the calcium free buffer is replaced by DMEM containing 3.3ng/ml aphidicolin and the neurons were incubated for 3 days at 37$^0$C in $CO_2$ incubator prior to electrophysiology recordings.

Immunofluorescence analysis showed that injection of antisense p11 resulted in a loss of immunoreactive p11 compared to uninjected cells.

*Molecular cloning of N-terminus Nav1.8 for binding assay to p11*

cDNA of the $Na_V1.8$'s N-terminus of α-subunit was cut into three fragments and cloned into pGEX-5X-1 vector (Amersham). The resulting constructs were named N1 (amino acids 1-25), N2 (a.a 26-50 ), and N3 (a.a 51-127).The primers used were designed to introduce an EcoRI restriction enzyme cut site in the 5' direction and NotI in the 3' direction. Forward primers: SNSI-F, 5'-GGAATTCATGGAGCTCCCCTTTGCG-3'

SNS I(N2)-F, 5'-GGAATTCAAGCAGATTGCTGCTCACCGC-3'

SNS I(N3)-F, 5-' GGAATTCCCCAGGCCTCAGCTGGACTTG-3'

Reverse primers:

SNS I(N1)-R, 5'-AATT**GCGGCCGC**CTCGATCTCTGCCAGTGACTC-3'

SNS I(N2)-R, 5'-AATT**GCGGCCGC**CTTCTCGCCCTTGTCCTCCTG-3'

I-R, 5'-AATT**GCGGCCGC**AGACGCTTTGATGGCTGT-3'

EcoRI sites are underlined. NotI sites are boldfaced. PCR was carried out using SNS I-F/SNS I(N1)-R, SNS I(N2)-F/SNS I(N2)-R, SNS I(N3)-F/I-R primer pairs for N1, N2, and N3 fragments respectively. The following cycles were used: 25 cycles of 1 min at 94°C, followed by 1 min 30 sec at 55°C, and then 1 min 30 sec at 72°C. PCR products were digested with EcoRI and NotI and ligated into linearized pGEX-5X-1 vector. The resulting constructs were sequenced with forward primer 5'pGEX (5'-GGGCTGGCAAGCCACGTTTGGTG-3') and reverse primer 3'pGEX (5'-CCGGGAGCTGCATGTGTCAGAGG-3').

The N3 fragment was further cut into three smaller fragments to elucidate the exact binding site on $Na_V1.8$ intracellular loop I after GST pulldown assay has shown that p11 binds specifically to N3. The three fragments, named N3-1(a.a 51-73), N3-2 (a.a 74-103) and N3-3 (a.a 104-127) were cloned into pGEX-5X-1 vector as described above.

Forward primers:

N3-1-5', 5'-GGAATTCCCCAGGCCTCAGCTGGACTTG-3'

N3-2-5', 5'-GGAATTCCTGGTCGGGGAGCCCCTGGAG-3'

N3-3-5', 5'-GGAATTCTTCAGTGCCACTTGGGCC-3'

Reverse primers:

N3-1-3', 5'-AATT**GCGGCCGC**TTCTGCTGGGAGCTC-3'

N3-2-3', 5'-AATT**GCGGCCGC**TCTGGAAATGGTCCTGCT-3'

PCR was carried out using N3-1-5'/N3-1-3', N3-2-5'/N3-2-3', N3-3-5'/I-R primer sets for N3-1, N3-2, and N303 respectively. The same PCR cycles were used as described above.

Example 1: Identification of a protein that interacts with rat Nav1.8

A rat sensory neuron cDNA library[11] was used to screen for proteins that interact with the N-terminal intracellular domain of rat $Na_V1.8$. Five identical positive clones encoding a full length p11 were identified through their interaction with the N-terminus of $Na_V1.8$. To test whether p11 binds to the N-terminal intracellular domain of $Na_V1.8$ *in vitro*, we expressed the GFP-p11 fusion protein in COS-7 cells, and expressed the N-terminal domain of $Na_V1.8$ as a GST fusion protein, GST-SNS(I). The COS cell lysates were incubated with affinity-purified GST and GST-SNS(I), the N-terminal domain of $Na_V1.8$ fused to GST, immobilized on glutathione-sepharose beads and were examined by immunoblotting with anti-GFP antibodies. Purified GST or GST/SNS (I) did not pull down GFP protein. Purified GST did not pull down GFP/SNS (I), while GST/p11 efficiently pulled down the GFP/SNS (I) fusion protein. These data demonstrate that p11 binds directly to the $NH_2$-terminal of SNS sodium channel.

We examined the tissue distribution of the p11 transcript. Northern blot analysis

showed high levels of expression of p11 mRNA in DRG, modest expression in heart and liver, and weak expression in brain isolated from 2 weeks old rats. RT-PCR showed a dramatic increase of p11 mRNA in cultured rat DRG neurons treated with nerve growth factor (NGF) which is known to cause decreases in thermal, chemical and mechanical thresholds of pain perception in animal models[13,14]. *In situ* hybridization was performed on a section of 2 weeks old rat DRG. An antisense p11 probe demonstrated strong staining in both small and large diameter neurons. Combined immunohistochemistry with anti-$Na_V1.8$ polyclonal antibody SNS11[15] showed that most (>98%) of the $Na_V1.8$ positive cells also expressed p11 mRNA.

Example 2 - p11 regulates translocation of SNS sodium channel into plasma membrane

CHO-SNS 22 cells are stably transfected cell line with rat SNS sodium channel cDNA. They do not have SNS sodium channel current however they express high amount of full length SNS sodium channel mRNA. Immunocytochemical study using anti-SNS sodium channel polyclonal antibody (SNS 11) showed SNS sodium channel-like immunoreactivity in cytosol of CHO-SNS 22 cells but not in the plasma membrane. To study whether p11 changes the cellular localization of SNS sodium channel protein, we transfected CHO-SNS 22 cells with GFP/p11 fusion cDNA (pBS-GFP/p11). The p11 expression was detected as green fluorescence signal due to fused GFP. The green fluorescence specifically localized in the plasma membrane. In the same cell, SNS sodium channel-like immunoreactivity, red fluorescence, also shows signal in the plasma membrane as well as cytosol. Co-expression of p11 and SNS sodium channel was seen in the plasma membrane. Densitometric analysis of $Na_V1.8$-like immunoreactivity of the GFP-p11 fusion or GFP protein expressed CHO-SNS22 cells showed that 16.5% (S.E.M. 1.2, n=30) of $Na_V1.8$-like immunoreactivity moved to the plasma membrane fraction after the expression of GFP-p11 fusion protein, while only 4.3% (S.E.M. 0.4, n=30) of $Na_V1.8$-like immunoreactivity localized on the plasma membrane in the GFP expressing CHO-SNS22 cells. These data demonstrate that p11 promotes the translocation of $Na_V1.8$ protein to the extracellular membrane.

Example 3 - p11 induces SNS sodium channel current in CHO-SNS 22 cells

In 9 from a total of 42 CHO-SNS 22 cells transfected with pBS-GFP/p11, TTX-resistant (TTX-r) currents were found that resembled neuronal SNS sodium channel current. The currents very closely resembled the SNS sodium channel $Na^+$ current recorded from transfected COS cells, both in terms of voltage-dependence and kinetics. The TTX-r inward currents are shown in Figure 1A. The current began to activate around 0 to +10 mV, and peaked at +40 mV (Fig. 1B). The reversal potential for the currents is quite close to the theoretical reversal potential for $Na^+$ (+81 mV), consistent with them being $Na^+$ currents. Any apparent deviation from the theoretical value may be considered within experimental error, given the small size of the inward current and the often non-linear current-voltage relations of CHO cells. No TTX-r inward currents have ever been recorded before from either non-transfected (n=41) or GFP transfected (n=40) CHO-SNS22 cells, following p11 transfection a little over 20 % of the cells generated small $Na^+$ currents. This is a highly statistically significant finding ($P < 0.002$ vs. GFP transfected or control non-transfected, Fisher exact test). This suggests that p11 may be a protein necessary for SNS sodium channel $Na^+$ channel function, normally missing from the CHO cell.

Nerve growth factor (NGF) is known as a potent hyperalgesic mediator[5]. On the basis of the results shown herein i.e. the involvement of p11 on the trafficking of SNS sodium channel protein into plasma membrane, it appears that the known function of NGF as a hyperalgesic mediator may be due to up-regulation of p11[7] and subsequent membrane translocation of SNS sodium channel by p11 (without altering the amount of SNS sodium channel mRNA[6]).

Prostaglandins such as prostaglandin E2 ($PGE_2$) act as hyperalgesic agents and its generation depends on cyclo-oxygenase (COX)-catalyzed conversion of arachidonic acid. The liberation of arachidonic acid from membrane glycerophospholipids is mediated by the hydrolytic action of phospholipase A2 and this is the rate-limiting step in the generation of prostaglandins. p11 is also known as an endogenous

inhibitor for cytosolic phospholipase A2 (cPLA2)[8]. Transforming growth factor-α (TGF-α) has been shown to stimulate expression of COX-2, cPLA2, and p11 in an epithelial cell line[9]. This suggests the expression of positive (COX-2, cPLA2) and negative (p11) regulator for prostaglandin synthesis is controlled by a common mechanism. Interestingly, calpactin-I heavy chain has antiflammin-like sequence which share a core tetrapeptide KVXD. Antiflammins are a peptides that share a common sequence with uteroglobin and lipocortin-I, which may act as anti-inflammatory agent by suppressing leukocyte trafficking to the lesion[10] however their activity in inflammation *in vivo* is still controversial.

Example 4: Effect of p11 antisense on Nav1.8 channels

To test the possible regulatory role of p11 on $Na_V1.8$ channels *in sensory neurons* , we microinjected the p11 antisense expression vector, pBS-GFP/AS(p11), into the nuclei of DRG neurons in culture. Immunohistochemistry, using anti-p11 polyclonal antibodies confirmed an efficient reduction of p11-like immunoreactivity in DRG neurons by the introduction of pBS-GFP/AS(p11). The introduction of pBS-GFP/AS(p11) also caused a dramatic loss of $Na_V1.8$ current (Figure 2). The mean peak $Na^+$ current density was reduced in pBS-GFP/AS(p11) injected neurons (63.1 +/- 24.5 pA/pF, mean +/- S.E.M. n=8) when compared with control neurons injected with GFP only (179.2 +/- 40.3 pA/pF, n=9, $P < 0.04$; Student's two tailed t-test). In contrast, the residual maximum $K^+$ current density derived from currents recorded on stepping to +80 mV were not significantly affected by pBS-GFP/AS(p11) (61.7 +/- 17.6 pA/pF in control cells vs. 44.8 +/- 5.5 pA/pF in injected, (means ±S.E.M., P = 0.4; Student's two tailed t-test), suggesting that the effect of antisense was specific. We also examined the affect of pBS-GFP/AS/p11 on TTX-sensitive currents in ND7/23 cells. TTX-sensitive current densities were unaffected ($p>0.1$) Students unpaired two-tailed t-test), suggesting that p11 is not required for the expression of other TTX-sensitive sodium channel subtypes.

The present inventors have demonstrated that the functional expression of the TTX-insensitive VGSC Nav 1.8 (which hereinafter may be referred to as the "SNS sodium

channel") is facilitated by interaction with a second protein - p11. This improved function appears to be effected through direct protein-protein interaction.

Example 5: Molecular cloning of p11 to elucidate binding domain to $Na_V1.8$

p11 was initially divided into three fragments (amino acids 1-32, a.a 33-77, and a.a 78-95) and cloned into pBS500 expression vector. The primers used were designed to introduce a NcoI restriction enzyme cut site in the 5' direction and XbaI in the 3' direction.

Forward primers: p11-5', 5'-AACCATGGATGCCATCCCAAATG-3';
p11-2-5', 5'- AACCATGGGTGCTCATGGAAAG-3';
p11-3-5', 5'- AACCATGGGGGCTCATCATTG-3'.
Reverse primers: p11-1-3', 5'-GA**TCTAGA**TCTCAGGTCCTCCTTTGTC-3';
p11-2-3', 5'- GA**TCTAGA**CGCCACTAGTGATAGAAAGC-3';
p11-3-3', 5'-GA**TCTAGA**CTACTTCTTCTGCTTCATGTGTAC-3'.
NcoI sites are underlined. XbaI sites are boldfaced. PCR was carried out using p11-5'/ p11-1-3', p11-2-5'/p11-2-3', p11-3-5'/p11-3-3" primer sets for fragments N3-1, N3-2, and N3-3 respectively. The following cycles were used: 25 cycles of 1 min at 94°C, followed by 1 min 30 sec at 55°C, and then 1 min 30 sec at 72°C. PCR products were digested with restriction enzymes EcoRI and NotI and ligated into pre-linearized pBS500 vector. The resulting constructs were sequenced with forward primer GFP5 (5'-ACCACATGGTCCTTCTTGAG-3') and reverse primer CSF-R (5'- TGCTGTTTAAATATTAAACAGGG-3').

The p11-2 fragment was further cut into two smaller fragments (amino acids 33-51 and 52-77) and cloned into pBS500 expression vector as described above. Forward primers: p11-2-2F, 5'-AACCATGGGTGGACAAAATAATGAAAGAC-3'; p11-2-5'. Reverse primers: p11-2-3'; p11-2-1R, 5'-GA**TCTAGA**AGCCAGAGGGTCCTTTTGA-3'. PCR was carried out using p11-2-5'/p11-2-1R and p11-2-2F/ p11-2-3' primer sets for fragment p11-2-1 and p11-2-2 respectively. Another fragment p11-1A (amino acids 1-51) was also cloned using the

primer set p11-5' and p11-2-1R.

GFP/p11 fusion protein was expressed in COS-7 cells by transient transfection. The $Na_V1.8$ N-terminal region was truncated into three separate fragments, fused to GST, immobilized on glutathione-sepharose beads and were examined by immunoblotting with anti-GFP antibodies. GFP/p11 binds directly to N3 region (aa 53-127). Purified GST-N1 and GST-N2 did not pull down GFP/p11 nor did GST control bind to GFP/p11.

GFP and GFP/p11 fusion protein were expressed in COS-7 cells by transient transfection. The COS cell lysates were incubated with affinity-purified GST-N3-1, -N3-2, and –N3-3 fragments of the N-terminal domain of $Na_V1.8$ fused to GST, immobilized on glutathione-sepharose beads and were examined by immunoblotting with anti-GFP antibodies. Purified GST-N3-1 and GST-N3-3 did not pull down GFP/p11, while GST--N3-2 efficiently pulled down the GFP/p11 fusion protein (amino aicds 75-102).

GFP/p11-1, -2, -3 fusion protein were expressed in COS-7 cells by transient transfection. The COS cell lysates were incubated with affinity-purified GST-SNS(I), the N-terminal domain of $Na_V1.8$ fused to GST, immobilized on glutathione-sepharose beads and were examined by immunoblotting with anti-GFP antibodies. GST-SNS(I) did not pull down cell lysates p11-1 and p11-3, but it efficiently pulled down cell lysate p11-2 (amino acids 33-77).

GFP/p11-1A, p11-2, p11-2-1, and p11-2-2 fusion proteins were expressed in COS-7 cells by transient transfection. The COS cell lysates were incubated with affinity-purified GST-SNS(I), the N-terminal domain of $Na_V1.8$ fused to GST, immobilized on glutathione-sepharose beads and were examined by immunoblotting with anti-GFP antibodies. GST-SNS(I) did not pull down cell lysates p11-1A, p11-2-1, nor p11-2-2. These protein fragments correspond to amino acids 1-51, 33-51, 52-77 respectively. GST-SNS(I) efficiently pulled down cell lysate p11-2.

Example 6: Mutagenesis of p11 Mutated-EF Hand

To delineate residues that are important for p11 to bind to $Na_V1.8$, point mutations were generated in the p11 EF hand binding region of construct p11-2. The oligonucleotide-directed point mutation was made by a two-step polymerase chain reaction protocol using two mutagenic primers and two restriction site primers. Full-length p11 in pBS500 expression vector was used as template. The following primer sets were used to introduce mutation 1 in amino acids 56 to 60 (from amino acid sequence DQCRD to AQARA): p11-5'/p11-mutation1-R; p11-3'/p11-mutation1-F. Forward primers: p11-5', 5'-AACCATGGATGCCATCCCAAATG-3'; p11-mutation1-F, 5'-GGCCCAGGCCCGAGCTG-3'. Reverse primers: p11-3', 5'-GATCTAGACTAC TTCTTCTGCTTCATGTGTAC-3'; p11-mutation1-R, 5'-TCCAGCTCGGGCCTGGGCC-3'. PCR cycles: 35 cycles of 94°C 1min, 63°C 1min 30sec, 72°C 1min 30 sec.

The following primer sets were used to introduce mutation 2 in amino acids 62 to 67 (from amino acid sequence KVGFQS to AVAFQA) in p11: p11-5'/p11-mutation2-R; p11-3'/p11-mutation2-F. Forward primer: p11-mutation2-F, 5'-GAGCAGTGGCCT TCCAGGCCT-3'. Reverse primer: p11-mutation2-R, 5'-TAGAAAGGCCTGGAA GGCCACTGCT-3'. PCR cycles: 40 cycles of 94°C 1min, 60°C 1min 30sec, 72°C 1min 30 sec.

The two PCR products containing the mutations were subcloned into pBS500 expression vector at NcoI/XbaI sites. Incorporation of the mutation was confirmed by DNA sequencing using the forward primer GFP5', 5'-ACCACATGGTCCTTCTTGAG-3'.

In summary, the data presented here show that the p11 is responsible for translocation of SNS sodium channel protein into plasma membrane and is a

necessary permissive factor for SNS sodium channel function. Therefore the interaction between p11 and SNS sodium channel can provide a target for therapeutic intervention in pain states.

References

1 Fitzgerald, E.M., Okuse, K., Wood, J.N., Dolphin, A.C., Moss, S.J. (1999) cAMP-dependent phosphorylation of the tetrodotoxin-resistant voltage-dependent sodium channel SNS sodium channel. *J. Physiology* 516 433-446.

2 Waisman, D.M. (1995) Annexin II tetramer: structure and function., *Mol. Cell. Biochem.* 149/150, 301-322.

3 Rety, S., Sopkova, J., Renouard, M., Osterloh, D., Gerke, V., Tabaries, S., Russo-Marie, F., Lewit-Bentley, A. (1999) The crystal structure of a complex of p11 with the annexin II N-terminal peptide., *Nat. Struct. Biol.* 6(1), 89-95.

4 Osborn, M., Johnsson, N., Wehland, J., Weber, K. (1988) The submembranous location of p11 and its interaction with the p36 substrate of $pp60^{src}$ kinase *in situ.*, *Exp. Cell. Res.* 175(1), 81-96.

5 McMahon, S.B., Bennet, D.L., Priestley, J.V., Shelton, D.L. (1995) The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule., *Nature Med.* 1(8), 774-780.

6 Okuse, K., Chaplan, S.R., McMahon, S.B., Luo, Z.D., Calcutt, N.A., Scott, B.P., Akopian, A.N., Wood, J.N. (1997) Regulation of expression of the sensory neuron-specific sodium channel SNS sodium channel in inflammatory and neuropathic pain., *Mol. Cell. Neurosci.* 10(3-4), 196-207.

7 Masiakowski, P., Shooter, E.M. (1988) Nerve growth factor induces the genes for two proteins related to a family of calcium-binding proteins in PC12 cells., Proc. *Natl. Acad. Sci. U.S.A.* 85(4), 1277-1281.

8 Wu, T., Angus, C.W., Yao, X.L., Logun, C., Shelhamer, J.H. (1997) P11, a unique member of the S100 family of calcium-binding proteins, interacts with and inhibits the activity of the 85-kDa cytosolic phospholipase A2., *J. Biol. Chem.* 272(27), 17145-17153.

9 Akiba, S., Hatazawa, R., Ono, K., Hayama, M., Matsui, H., Sato, T. (2000)

Transforming growth factor-α stimulates prostaglandin generation through cytosolic phospholipase A2 under the control of p11 in rat gastric epithelial cells., *Br. J. Pharmacol.* 131, 1004-1010.

10 Moreno JJ. (2000) Antiflammin peptides in the regulation of inflammatory response., *Ann. N.Y. Acad. Sci.* 923, 47-53.

11 Kong, H. et al. An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. *J. Neurosci.* **21**, 176-185 (2001).

12 Liu, C.j., Dib-Hajj, S.D., Waxman, S.G. Fibroblast growth factor homologous factor 1B binds to the C terminus of the tetrodotoxin-resistant sodium channel rNav1.9a (NaN). J. Biol. Chem. **276**, 18925-18933 (2001).

13 Lewin, G.R., Rueff, A. & Mendell, L.M. Peripheral and central mechanisms of NGF-induced hyperalgesia. *Eur. J. Neurosci.* **6**, 1903-1912. (19 )

14 Black JA et al. NGF has opposing effects on Na+ channel III and SNS gene expression in spinal sensory neurons. *Neuroreport.;8, 2331-5. (1997)*

15 Fang, X. et al. Sensory and electrophysiological properties of DRG neurones with SNS-like immunoreactivity (SNS-LI) in rats. *Soc. Neurosci. Abstr.* **819.5** (2001).

16 Fjell, J. et al. In vivo NGF deprivation reduces SNS expression and TTX-R sodium currents in IB4-negative DRG neurons. J. Neurophysiol. **81**, 803-810 (1999).

CLAIMS

1. A method of identifying a modulator of a voltage gated sodium channel (VGSC), which method comprises:

(a) bringing into contact a VGSC, a p11 peptide and a test compound under conditions where the VGSC and the p11 peptide are capable of forming a complex in the absence of the test compound; and

(b) measuring an activity of the VGSC, wherein a change in the activity of the VGSC relative to the activity in the absence of the test compound indicates that the test compound is a modulator of said VGSC.

2. A method according to claim 1 wherein said activity is the ability of the VGSC to form a complex with the p11 peptide.

3. A method according to claim 1 wherein said activity is the ability of the VGSC to mediate a sodium current across a membrane.

4. A method according to any one of the preceding claims wherein a decrease in the activity of the VGSC indicates that the test compound is an inhibitor of said VGSC.

5. A method according to any one of the preceding claims wherein said VGSC is a channel associated with responses to pain.

6. A method according to any one of the preceding claims wherein said channel is expressed in sensory neurons.

7. A method according to claim 6 wherein said channel is sensory neuron specific (SNS).

8. A method according to any one of the preceding claims wherein said channel

is tetrodotoxin resistant.

9. A method according to any one of claims 1 to 6 wherein said VGSC is selected from the Nav 1.8, Nav 1.9 and Nav 1.3 sodium channels.

10. A method according to any one of the preceding claims wherein said VGSC has an amino acid sequence comprising:

(a) the Nav 1.8 amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 6;

(b) a species or allelic variant of (a);

(c) a variant of (a) having at least 65% amino acid sequence identity thereto; or

(d) a fragment of any of (a) to (c);

wherein said VGSC retains the ability to bind a p11 peptide.

11. A method according to claim 10 wherein said VGSC retains the ability to mediate a sodium current across a membrane.

12. A method according to claim 10 or 11 wherein said VGSC comprises the sequence of amino acids 53 to 127 or 75 to 102 of SEQ ID NO: 2.

13. A method according to any one of the preceding claims wherein said p11 peptide has an amino acid sequence comprising:

(a) the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 8;

(b) a species or allelic variant of (a);

(c) a variant of (a) having at least 70% amino acid sequence identity thereto; or

(d) a fragment of any of (a) to (c);

wherein said p11 peptide retains the ability to bind a VGSC.

14. A method according to claim 13 wherein said p11 peptide comprises the sequence of amino acids 33 to 77 of SEQ ID NO 4.

15. A method according to any one of the preceding claims wherein said p11

peptide is a full length p11 protein or a species or allelic variant thereof.

16. A method according to any one of the preceding claims wherein said VGSC and said p11 peptide are provided in a cell and said cell is contacted with a test compound.

17. A method according to any one of the preceding claims wherein said VGSC is provided in a cell in which the functional expression of said channel has been enhanced by increasing the level of p11 in the cell.

18. A method according to claim 16 or 17 wherein said cell endogenously expresses said VGSC and/or said p11 peptide

19. A method according to claim 16 or 17 wherein said channel and/or said p11 peptide are expressed from one or more vectors introduced into said cell.

20. A method according to claim 1 comprising the steps of:

(i) providing a cell in which the functional activity of an SNS sodium channel has been enhanced by increasing the concentration of p11 in the cell;

(ii) contacting the channel in the cell with the test compound; and

(iii) measuring the activity of the channel.

21. A method according to claim 1 comprising the steps of:

(i) bringing into contact an SNS sodium channel, p11, and a putative modulator compound under conditions where the SNS sodium channel and the p11, in the absence of modulator, are capable of forming a complex; and

(ii) measuring the degree of inhibition of complex formation baused by said modulator compound.

22. A method according to claim 1 comprising the steps of:

(i) bringing into contact an SNS sodium channel, p11, and a putative modulator

compound under conditions where the SNS sodium channel and the p11, in the absence of modulator, are capable of forming a complex;

(ii) exposing the SNS sodium channel to a stimulus such as to produce a sodium current across a membrane in which the SNS sodium channel is present; and

(iii) measuring the degree of inhibition of the current caused by said modulator compound.

23. A method according to any one of the preceding claims further comprising the step of formulating said test compound as a pharmaceutical composition.

24. A method according to claim 23 further comprising administering said formulation to an individual for the treatment of pain.

25. A method of enhancing the functional expression of a voltage gated sodium channel (VGSC) in a cell which method comprises the step of increasing the level of p11 in the cell.

26. A method according to claim 25 wherein said VGSC is as defined in any one of claims 5 to 12.

27. A method according to claim 25 or 26 wherein said p11 is as defined in any one of claims 13 to 15.

28. A compound identified by a method of any one of claims 1 to 24.

29. A host cell capable of expressing a VGSC and a p11 peptide wherein said VGSC and/or said p11 peptide is expressed from one or more heterologous expression vectors within said cell.

30. Use of a compound identified by a method of any one of claims 1 to 24 in the manufacture of a medicament for modulating the functional expression of a voltage

gated sodium channel.

31. Use of an inhibitor of p11 activity or expression in the manufacture of a medicament for modulating the functional expression of a voltage gated sodium channel.

32. Use according to claim 30 or 31 wherein said medicament is for producing analgesia.

33. Use according to claim 30, 31 or 32 wherein said medicament is for relieving chronic pain.

34. Use according to any one of claims 30 to 33 wherein said inhibitor of p11 activity or expression is selected from an antibody or fragment thereof specific to the p11 peptide and antisense cDNA directed to the sequence encoding the p11 peptide.

35. Use according to claim 34 wherein said antibody or fragment thereof is directed to an epitope in the region of a p11 protein defined by amino acids 33 to 77 of SEQ ID NO: 4.

36. A method of treating a disorder or condition associated with the activity of a voltage gated sodium channel, said method comprising administering to an individual in need thereof a compound identified by a method of any one of claims 1 to 24 or an inhibitor of p11 activity or expression.

37. A peptide comprising at least 10 contiguous amino acids of
(a) the sequence from amino acid 75 to amino acid 102 of SEQ ID NO: 2, or
(b) a sequence having at least 65% amino acid sequence identity to (a),
wherein said peptide is capable of specifically binding a p11 peptide and is less than 1000 amino acids in length.

38. A peptide comprising at least 10 contiguous amino acids of
(a) the sequence from amino acid 33 to amino acid 77 of SEQ ID NO: 4, or
(b) a sequence having at least 70% amino acid sequence identity to (a),
wherein said peptide is capable of specifically binding a voltage gated sodium channel and is less than 80 amino acids in length.

39. A peptide according to claim 37 or 38 which comprises at least 15, 20 or 25 contiguous amino acids of (a) or (b).