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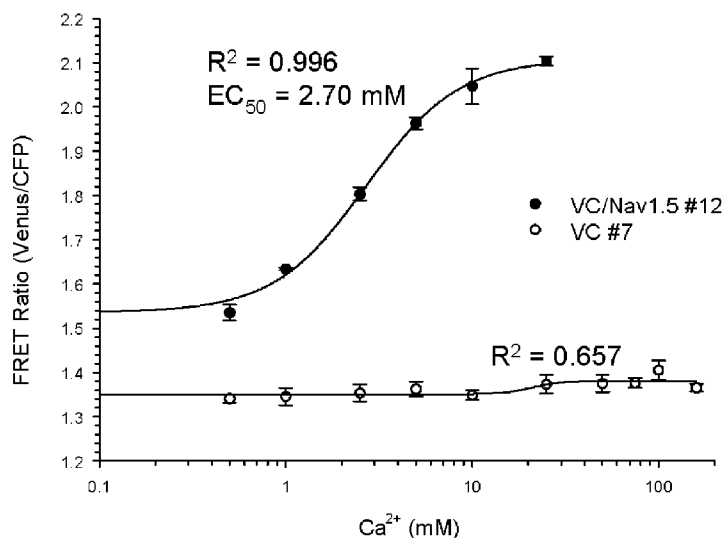


Fig. 5

(57) Abstract: Reagents, methods and kits for screening for compounds that modulate the activity of voltage-gated sodium channels (NaV), such as human NaV1.5/SCN5A/hH1 are described. The reagents, methods and kits are based on mutated NaV alpha subunit polypeptides of SEQ ID NO:5 with mutations at positions 372, 898, 1419 and 1711 (the DEKA motif) and at positions 11485, 1486 and 1487 (the IFM motif) resulting in increased permeability for a group IIA divalent cation (Ca²⁺) and decreased inactivation rate. The mutant polypeptide is used in a method and kit for determining whether a test compound modulates the channel activity, preferably using a chimeric polypeptide (chameleon polypeptide) comprising calmodulin, a calmodulin binding protein (M13), and two fluorescent agents.

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MUTATED VOLTAGE-GATED SODIUM CHANNEL NAV ALPHA SUBUNIT FOR IDENTIFICATION OF MODULATORS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Patent Application serial No. 61/139,790 filed on December 22, 2008, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present invention generally relates to sodium channels. More specifically, the present invention relates to the identification of modulators of voltage-gated sodium channels (Na_v), such as human Na_v1.5.

BACKGROUND ART

Voltage-gated sodium channels (VGSC or Na_v) produce the rapid upstroke of the action potential and are important elements for maintaining electrical excitability and assuring the coordination of excitation-contraction coupling in striated muscle and neuronal excitability. As shown in Fig. 1, they are composed of one α -subunit (260 kDa in the example shown), which forms the core of the channel and which is responsible for the voltage-dependent gating and ion permeation (Catterall WA, *Annu Rev Biochem* 1986, **55**: 953-985; Fozzard HA and Hanck DA, *Physiol Rev* 1996, **76**: 887-926; Armstrong CM and Hille B, *Neuron* 1998, **20**: 371-380). The α -subunit is composed of four homologous domains (DI-DIV), each with six α -helical transmembrane-spanning segments (S1-S6). The S1-S4 domains form the voltage sensor domains (Stühmer W *et al.*, *Nature* 1989, **339**: 597-603; Yang N *et al.*, *Biophys J* 1997, **73**: 2260-2268; Kontis KJ *et al.*, *J Gen Physiol* 1997, **110**: 391-401). The short linkers connecting the S5 and S6 segments form the external mouth of the pore and the selective filter (Pérez-García MT *et al.*, *Biophys J* 1997, **72**: 989-996; Yamagishi T *et al.*, *Biophys J* 1997, **73**: 195-204; Chiamvimonvat N *et al.*, *Neuron* 1996, **16**: 1037-1047; Pérez-García MT *et al.*, *Proc Natl Acad Sci USA* 1996, **93**: 300-304). The cytoplasmic linker between the third (DIII) and fourth (DIV) homologous domains acts as a "hinged lid" that occludes the internal end of the permeation pathway during inactivation (Stühmer W *et al.*, 1989, *supra*; Armstrong CM and Bezanilla F, *J Gen Physiol* 1977, **70**: 567-590; West JW *et al.*, *Proc Natl Acad Sci USA* 1992, **89**: 10910-10914). Residues of the S6 segments from each of the four homologous domains (DIS6-DIVS6) line the internal vestibule and contribute to the binding site for local anaesthetics (LA) and antiarrhythmic drugs (Ragsdale DS *et al.*, *Proc Natl Acad Sci USA* 1996, **93**: 9270-9275). The cytoplasmic ends of the S6 segments and the short linkers from each of the four

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homologous domains that connect the S4-S5 segments contribute to the binding site for the native inactivation gate (Smith MR and Goldin AL, *Biophys J* 1997, **73**: 1885-1895; McPhee JC *et al.*, *Proc Natl Acad Sci USA* 1994, **91**: 12346-12350; McPhee JC *et al.*, *J Biol Chem* 1995, **270**: 12025-12034).

5 Structure-function studies indicated that the S5-S6 linkers constitute the pore-forming regions known as P-loops of the channel (Pérez-García MT *et al.*, 1996, *supra*; Heinemann SH *et al.*, *Nature* 1992, **356**: 441-443; Terlau H *et al.*, *FEBS Lett* 1991, **293**: 93-96). Each P loop is composed of two short segments called SS1 and SS2, for short segment 1 and short segment 2 respectively, they span part of the plasma membrane (Terlau H *et al.*, 1991, 10 *supra*; Guy HR and Conti F, *Trends Neurosci* 1990, **13**: 201-206; Guy HR and Seetharamulu P, *Proc Natl Acad Sci USA* 1986, **83**: 508-512).

Sodium (Na) channel blockers have been developed and used for therapeutic purposes for several decades. One of the earliest compounds used for therapeutic purposes, that was later shown to block Na channels is cocaine. Cocaine, an aminoester, was the first 15 local anesthetic drug useful in clinical surgery but it had undesirable side effects. It was however soon realized that the anesthetic properties of cocaine were preserved in chemically similar structures that had less undesirable side effects. This quickly led to the development of an entire class of cocaine-related compounds comprising other aminoesters like benzocaine and procaine, as well as aminoamides, like bupivacaine and lidocaine. Most of these drugs were/are 20 typically not administered orally, but topically or intrathecally, thereby preventing adverse side effects, like convulsions and cardiovascular collapse, still associated with these drugs when applied systemically. The mechanisms by which these compounds interact with Na channels have been the subject of many scientific studies. Lidocaine in particular, still widely used in the clinic today, has been studied extensively. Lidocaine has shown efficacy in numerous pain 25 conditions including diabetic neuropathy and postherpetic neuralgia.

Besides the "caine" class of analgesic compounds, certain clinically used anticonvulsants, antidepressants, and antiarrhythmics have inhibitory activity on Na channels, which at least partially, underlie their clinical efficacy. Among such compounds are phenytoin, carbamazepine, and aminotriptyline. All these drugs block Na channels by binding to the DI-IV 30 S6 transmembrane helices.

More recently, a number of mutations have been found to cause abnormal Na channel functions leading to human diseases or Na channelopathies such as periodic paralysis, myotonia, long QT syndrome and other cardiac conductance disturbances, pain, and epilepsy George AL Jr., *J Clin Invest* 2005, **115**: 1990-1999).

35 Conventional methods for assaying sodium channel activity include radiolabeled toxin-binding assays, radioactive ion influx assays, electrophysiological patch-clamp assays,

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and membrane potential dyes (Reviewed by Terstappen, 2005, *Drug Discov Today: Technologies* 2(2): 133-40). All these assays have major disadvantages that limit their use. For example: i) the radioactive ion influx method requires long incubation time and multiple wash steps, necessitating non-homogeneous assay format. Moreover, it requires the use of chemical modifier of channel inactivation, introducing the risk of false positive or false negative results, and finally, it produces a large quantity of costly radioactive waste; ii) The patch clamp technique, largely considered the "gold standard", has inherent limitations, including low throughput and specialized equipment incompatible with standard laboratory robotics. Although higher throughput can be achieved with higher throughput patch-clamp such as IonWorks™ or PatchXpress™ (both from Molecular Devices Inc.), these assays are still relatively expensive and not well adapted for fast kinetics of VGSC.

Currently, in the industry, another approach that is used for drug-screening assays with VGSC is based on membrane potential-sensitive fluorescent dyes, such as bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC₄(3)), because there are no efficient sodium dyes available. However, three major problems are associated with this technology: (i) Dyes such as DiBAC₄(3) are sensible to any membrane potential changes and as a result it is not possible to employ extracellular potassium to open sodium channels and measure the sodium influx. It is necessary to use toxins, such as veratridine which the mechanism of action is not yet elucidated, to activate the sodium channel and monitor the ion influx. In a high-throughput screening context, this can generate many false positive or false negative results because the direct binding of veratridine to channels clearly changes their native conformation, and probably alters the interaction with the compounds to be tested; (ii) the use of fluorescent dyes involves time-consuming wash steps as well as the loss of cells and signal; (iii) membrane potential-sensitive fluorescent dyes are expensive, and are not suitable for endogenous expression due to their low response time and low sensitivity (Reviewed by Terstappen, 2005, *supra*).

There is thus a need for the development of novel reagents and methods for the identification of sodium channel modulators.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a mutated voltage gated sodium channel (Na_v) alpha subunit polypeptide comprising a first mutation that increases the permeability of the channel for a group IIA divalent cation and a second mutation that decreases the inactivation rate of the channel.

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In an embodiment, the above-mentioned first mutation is a mutation in a domain connecting a S5 and a S6 α -helical transmembrane-spanning segment. In a further embodiment, the above-mentioned first mutation is:

- 5 (i) a mutation at a residue corresponding to residue 372 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
- (ii) a mutation at a residue corresponding to residue 898 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
- (iii) a mutation at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
- 10 (iv) a mutation at a residue corresponding to residue 1711 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit; or
- (v) any combination of (i) to (iv).

In a further embodiment, the above-mentioned first mutation is a mutation at a residue corresponding to residue 1419 in the amino acid sequence of Na_v1.5 alpha subunit. In a further embodiment, the above-mentioned mutation at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit is a substitution to an alanine residue.

In an embodiment, the above-mentioned second mutation is a mutation in an intracellular region connecting the S6 α -helical transmembrane-spanning segment of Domain III and the S1 α -helical transmembrane-spanning segment of Domain IV.

In an embodiment, the above-mentioned second mutation is:

- (i) a mutation at a residue corresponding to residue 1485 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
- (ii) a mutation at a residue corresponding to residue 1486 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
- 25 (iii) a mutation at a residue corresponding to residue 1487 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit; or
- (iv) any combination of (i) to (iii).

In a further embodiment, the above-mentioned second mutation is a mutation at a residue corresponding to residue 1486 in the amino acid sequence of Na_v1.5 alpha subunit. In a further embodiment, the above-mentioned mutation at a residue corresponding to residue 1486 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit is a substitution to a glutamine residue.

In an embodiment, the above-mentioned first mutation is at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human Nav1.5 alpha

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subunit, and the above-mentioned second mutation is at a residue corresponding to residue 1486 in the amino acid sequence of isoform A of human Nav1.5 alpha subunit.

In an embodiment, the above-mentioned mutated Na_v alpha subunit polypeptide is a mutated human Na_v1.5 alpha subunit polypeptide. In a further embodiment, the above-mentioned mutated Na_v alpha subunit polypeptide comprises the amino acid sequence of SEQ ID NO: 5.

In an embodiment, the above-mentioned group IIA divalent cation is calcium (Ca²⁺).

In another aspect, the present invention provides an isolated nucleic acid encoding the above-mentioned mutated Na_v alpha subunit polypeptide. In an embodiment, the above-mentioned nucleic acid comprises the nucleotide sequence of SEQ ID NO: 1, 2, 3 or 4.

In another aspect, the present invention provides a vector comprising the above-mentioned nucleic acid.

In another aspect, the present invention provides a cell comprising the above-mentioned nucleic acid or vector.

In another aspect, the present invention provides a method for determining whether a test compound modulates the activity of a voltage gated sodium channel, said method comprising

(a) providing a cell expressing the above-mentioned mutated Na_v alpha subunit polypeptide; and

(b) determining the intracellular level of a group IIA divalent cation in said cell in the presence or absence of said test compound,

wherein a difference in the intracellular level of said group IIA divalent cation in the presence relative to the absence of said test compound is indicative that said test compound modulates the activity of a voltage gated sodium channel.

In an embodiment, the above-mentioned method further comprises providing a source of said group IIA divalent cation in the extracellular medium. In another embodiment, the above-mentioned group IIA divalent cation is calcium (Ca²⁺).

In an embodiment, the intracellular calcium level is determined by determining the conformational change of a calcium-binding polypeptide.

In an embodiment, the above-mentioned cell further expresses a first and second chimeric polypeptide, wherein

(i) said first chimeric polypeptide comprises:

(a) a first domain comprising a first fluorescent agent having an emission spectra;

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- (b) a second domain linked to said first domain and comprising a calcium-binding polypeptide;
- (ii) said second chimeric polypeptide comprises:
- (a) a first domain comprising a polypeptide which binds in a calcium-dependent manner to said calcium-binding polypeptide; and
- (b) a second domain linked to said first domain of said second chimeric polypeptide and comprising a second fluorescent agent having an absorption spectra which overlaps with the emission spectra of said first fluorescent agent;

wherein the intracellular calcium level is determined by measuring the intensity of the fluorescence emitted by said second fluorescent agent.

In another embodiment, the above-mentioned cell further expresses a first and second chimeric polypeptide, wherein

- (i) said first chimeric polypeptide comprises:
- (a) a first domain comprising a first fluorescent agent having an absorption spectra;
- (b) a second domain linked to said first domain and comprising a calcium-binding polypeptide;
- (ii) said second chimeric polypeptide comprises:
- (a) a first domain comprising a polypeptide which binds in a calcium-dependent manner to said calcium-binding polypeptide; and
- (b) a second domain linked to said first domain of said second chimeric polypeptide and comprising a second fluorescent agent having an emission spectra which overlaps with the absorption spectra of said first fluorescent agent; or

wherein the intracellular calcium level is determined by measuring the intensity of the fluorescence emitted by said first fluorescent agent.

In an embodiment, the above-mentioned calcium-binding polypeptide is Calmodulin (CaM) or a calcium-binding fragment thereof.

In an embodiment, the above-mentioned polypeptide binding in a calcium-dependent manner to said calcium-binding polypeptide is myosin light chain kinase or a calmodulin-binding domain thereof. In a further embodiment, the above-mentioned calmodulin-binding domain of a myosin light chain kinase comprises a domain corresponding to residues 577 to 602 of rabbit skeletal muscle myosin light chain kinase (M13). In a further embodiment, the above-mentioned calmodulin-binding domain of a myosin light chain kinase comprises the amino acid sequence of SEQ ID NO: 14.

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In an embodiment, the above-mentioned first fluorescent agent is a cyan fluorescent protein (CFP) and said second fluorescent agent is a yellow fluorescent polypeptide (YFP). In another embodiment, the above-mentioned first fluorescent agent is a yellow fluorescent polypeptide (YFP) and said second fluorescent agent is a cyan fluorescent protein (CFP).

In an embodiment, the above-mentioned second domain of said first chimeric polypeptide is linked to said first domain of said second chimeric polypeptide.

In an embodiment, the above-mentioned first and second chimeric polypeptides are recombinantly expressed as a single chimeric polypeptide. In a further embodiment, the above-mentioned single chimeric polypeptide comprises an amino acid sequence of a Cameleon polypeptide.

In an embodiment, the above-mentioned cell further comprises a second nucleic acid encoding the above-mentioned first and second chimeric polypeptides.

In another aspect, the present invention provides a kit comprising the above-mentioned cell and a container.

In another aspect, the present invention provides a kit comprising the above-mentioned vector and a container.

In another aspect, the present invention provides a kit comprising the above-mentioned vector, wherein said vector further comprises the above-mentioned second nucleic acid, and a container.

In another aspect, the present invention provides a kit comprising the above-mentioned vector, a second vector comprising the above-mentioned second nucleic acid, and a container.

In an embodiment, the above-mentioned kit further comprises instructions setting forth the above-mentioned method.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

In the appended drawings:

Fig. 1 shows the schematic representation of the general structure of the alpha (α) subunit of voltage-gated sodium channels. The α subunit folds into four domains (I to IV), each containing six α -helical transmembrane segments (1-6). Segments 5 and 6 are the pore-lining segments and the S4 helices, which contain positively charged amino acid residues in every third position, constitute the voltage sensors. "Gly" indicates putative N-linked

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glycosylation sites. The circles in the intracellular loops of domains III and IV indicate the inactivation gate "IFM" motif (h, inactivation gate). The "P" represent phosphorylation sites, with the circles representing sites for protein kinase A and the diamonds representing sites for protein kinase C. The location of the residues forming the aspartate-glutamate-lysine-alanine ("DEKA") ring are highlighted. An II = Annexin II; AnkG = Ankyrin G; CaM = Calmodulin; G β γ = G-protein β - γ complex; Ub = Ubiquitin;

Fig. 2 shows representative trace currents of HEK293 cells transiently transfected with Na_v1.5^{Mut} sodium channel. The currents were recorded in presence of 140 mM NaCl and 1.5 mM CaCl₂ in the extracellular solution (**Fig. 2A**), or with 95 mM CaCl₂ and 0 mM NaCl in the extracellular solution (**Fig. 2B**). Cells were held at -120mV and depolarized from -80 to +40 mV in 10 mV increments;

Fig. 3 shows representative confocal microscopy experiments involving the HEK293 Cameleon cell line. **Fig. 3A:** Individual Venus (right panel) and CFP (left panel) fluorescence image taken with a 40X objective. **Fig. 3B:** Plot of FRET ratio (Venus/CFP) versus time following addition of 10 μ M ionomycin;

Fig. 4 shows validation of the Na_v1.5^{Mut}/Cameleon stable cell lines. **Fig. 4A** shows a Z' evaluation of two Na_v1.5^{Mut}/Cameleon cell lines, VC/Na_v1.5 #12 (Z' = 0.72) and #14 (Z' = 0.69), and a control Cameleon cell line, VC #7 (Z' = 0.88). **Fig. 4B** shows a treatment of the Na_v1.5^{Mut}/Cameleon and Cameleon cell lines with ionomycin (grey bars) or veratridine (white bars);

Fig. 5 shows dose-response measurements of FRET signals in response to calcium. VC/Na_v1.5 #12 (filled dots) and control VC #7 (empty dots) cell lines were exposed to different extracellular calcium concentrations ([Ca²⁺]). The Cameleon was able to detect different [Ca²⁺] and VC/Na_v1.5 #12 showed a strong dose-response relationship ($R^2 = 0.996$). These results were obtained in fluorescence microplate assay with an excitation at 425 nm and emission at 480 nm (CFP) and 525 nm (Venus). The FRET ratio represents the ratio of the signal detected at 525 nm (Venus) over that detected at 480 nm (CFP). Error bars represent the standard deviation of experiments performed in triplicate;

Fig. 6 shows dose-response measurements of FRET signal in the presence of a sodium channel blockers. VC/Na_v1.5 #12 cells were incubated with different concentrations of quinidine (**Fig. 6A**), sertraline (**Fig. 6B**) or paroxetine (**Fig. 6C**), and a first fluorescence reading was performed, (F_{Venus}/F_{CFP})₀. 25 mM Ca²⁺ was added to activate the channel and a second fluorescence reading was performed, (F_{Venus}/F_{CFP})_{Ca2+}. These results were obtained in fluorescence microplate assay with an excitation at 425 nm and emissions at 480 nm (for F_{CFP}) and 525 nm (for F_{Venus}). The results are represented as the Delta FRET ratio, which is

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$(F_{\text{Venus}}/F_{\text{CFP}})_{\text{Ca}^{2+}} / (F_{\text{Venus}}/F_{\text{CFP}})_0$, as a function of sodium channel blocker concentration. Error bars represent the standard deviation of experiments performed in triplicate;

Fig. 7A shows the amino acid sequence of a mutated human Nav1.5 polypeptide ($\text{Na}_v1.5^{\text{mut}}$). The residues corresponding to the “DEKA” and “IFM” motifs are in bold and underlined, and the mutations relative to native human Nav1.5 are highlighted in grey (K to C at position 1419 and F to Q at position 1486); and

Figs. 7B-7E shows the amino acid sequences of various human $\text{Na}_v1.5$ alpha subunit polypeptides: $\text{Na}_v1.5$ isoform a (**Fig. 7B**), $\text{Na}_v1.5$ isoform b (**Fig. 7C**), $\text{Na}_v1.5$ isoform c (**Fig. 7D**), and $\text{Na}_v1.5$ isoform d (**Fig. 7E**). The residues corresponding to the “DEKA” and “IFM” motifs are in bold and underlined.

DISCLOSURE OF INVENTION

In the studies described herein, it is shown that cells expressing (i) a mutated $\text{Na}_v1.5$ channel having (a) an increased permeability for group IIA divalent cations (e.g., Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+}) and (b) a decreased inactivation rate, and (ii) a reporter system sensitive to modulations in divalent cation concentrations, may be used to screen for compounds that modulate Na_v channel activity.

Accordingly, in a first aspect, the present invention provides a mutated voltage gated sodium channel (Na_v) alpha subunit polypeptide comprising a first mutation that increases the permeability of the channel for a group IIA divalent cation and a second mutation that decreases the inactivation rate of the channel.

In an embodiment, the above-mentioned first mutation is a mutation in a domain connecting a S5 and a S6 α -helical transmembrane-spanning segment (see Fig. 1).

In an embodiment, the above-mentioned first mutation is:

- (i) a mutation at a residue corresponding to residue 372 in the amino acid sequence of isoform A of human $\text{Na}_v1.5$ alpha subunit;
- (ii) a mutation at a residue corresponding to residue 898 in the amino acid sequence of isoform A of human $\text{Na}_v1.5$ alpha subunit;
- (iii) a mutation at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human $\text{Na}_v1.5$ alpha subunit;
- (iv) a mutation at a residue corresponding to residue 1711 in the amino acid sequence of isoform A of human $\text{Na}_v1.5$ alpha subunit; or
- (v) any combination of (i) to (iv).

In an embodiment, the above-mentioned mutation at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human $\text{Na}_v1.5$ alpha subunit is a substitution to an alanine residue.

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In an embodiment, the above-mentioned second mutation is a mutation in an intracellular region connecting the S6 α -helical transmembrane-spanning segment of Domain III and the S1 α -helical transmembrane-spanning segment of domain IV (see Fig. 1).

In an embodiment, the above-mentioned second mutation is:

- (i) a mutation at a residue corresponding to residue 1485 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
- (ii) a mutation at a residue corresponding to residue 1486 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
- (iii) a mutation at a residue corresponding to residue 1487 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit; or
- (iv) any combination of (i) to (iii).

Correspondence of amino acid positions described herein is frequently expressed in terms of the positions of the amino acids of isoform A of the human Na_v1.5 alpha subunit polypeptide set forth in Fig. 7A (also called hH1, Gellens *et al.*, *Proc. Natl. Acad. Sci. USA* **89**: 554-558). For example, the positions correspond to one or more of the amino acids aspartic acid (D), glutamic acid (E), lysine (K) and alanine (A) ("DEKA" motif) set forth at positions 372, 898, 1419 and 1711, respectively, in the amino acid sequence depicted in Fig. 7A, or to one or more of the amino acids isoleucine (I), phenylalanine (F), and methionine (M) ("IFM" motif) set forth at positions 1485, 1486 and 1487, respectively, in the amino acid sequence depicted in Fig. 7A. A "mutated" or "modified" Na_v alpha subunit polypeptide as used herein refers to a polypeptide having voltage-gated ion channel activity (e.g., a truncated form of a Na_v alpha subunit polypeptide, a fusion polypeptide having voltage-gated ion channel activity, a chimeric polypeptide having ion channel activity, etc.), the polypeptide having (i) a different amino acid from the native protein at at least one of the amino acid positions described more fully in the specification, in relation to a wild-type or native Na_v polypeptide, (ii) an increased permeability for a group IIA divalent cation (e.g., Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺) relative to a native Na_v polypeptide and (iii) a decreased inactivation rate relative to a native Na_v polypeptide.

"Mutated" or "mutation" as used herein, for example in reference to a polypeptide or nucleic acid, refers to an altered version or alteration therein, relative to the native, naturally occurring (typically referred to as "wild-type") version of the polypeptide or nucleic acid. Mutations include for example substitution(s), insertion(s) and/or deletions of residue(s) (amino acid or nucleotide, as applicable) in a polypeptide or nucleic acid sequence.

Voltage-dependent sodium channels have been identified in a variety of animals, including flies, leeches, squid and jellyfish, as well as mammalian and non-mammalian vertebrates. These various channels share significant homology, particularly in the transmembrane and extracellular domains. For example, the mammalian sodium channel

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isoforms that have been identified and functionally expressed are all greater than 50% identical in amino acid sequence in the transmembrane and extracellular domains. More particularly, the “DEKA” and “IFM” motifs noted above are highly conserved among the various Na_v channels. It will be understood that amino acid numbering can thus be shifted in situations where the residues corresponding to DEKA and IFM residues in isoform A of the human Na_v1.5 alpha subunit polypeptide are within a polypeptide having more or fewer amino acids N-terminal to the region(s) where these residues reside, relative to isoform A of the human Na_v1.5 alpha subunit polypeptide, thereby resulting in different amino acid numbering relative to the positions of isoform A of the human Na_v1.5 alpha subunit polypeptide. The corresponding positions may be easily identified, for example by aligning the amino acid sequence of a given Na_v alpha subunit polypeptide with that of isoform A of the human Na_v1.5 alpha subunit polypeptide (e.g., using a software for sequence alignment such as Clustal W). For example, the positions corresponding to positions 372, 898, 1419 and 1711 of isoform A of the human Na_v1.5 alpha subunit polypeptide in other isoforms of human Na_v1.5 alpha subunit channels (Fig. 7C-7E) are depicted in Table I below, and the positions corresponding to positions 1485, 1486 and 1487 of isoform A of the human Na_v1.5 alpha subunit polypeptide in other isoforms of human Na_v1.5 alpha subunit channels are depicted in Table II below.

Table I: Positions corresponding to positions 372, 898, 1419 and 1711 of isoform A of the human Na_v1.5 alpha subunit polypeptide in other isoforms of human Na_v1.5 alpha subunit channels

Sodium Channel	Position corresponding to position 372 of isoform A of human Na _v 1.5 alpha subunit polypeptide	Position corresponding to position 898 of isoform A of human Na _v 1.5 alpha subunit polypeptide	Position corresponding to position 1419 of isoform A of human Na _v 1.5 alpha subunit polypeptide	Position corresponding to position 1711 of isoform A of human Na _v 1.5 alpha subunit polypeptide
Human Na _v 1.5, isoform b	372	898	1418	1710
Human Na _v 1.5, isoform c	372	898	1419	1711
Human Na _v 1.5, isoform d	372	898	1399	1693

Table II: Positions corresponding to positions 1485, 1486 and 1487 of isoform A of the human Na_v1.5 alpha subunit polypeptide in other isoforms of human Na_v1.5 alpha subunit channels

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Sodium Channel	Position corresponding to position 1485 of isoform A of human Na _v 1.5 alpha subunit polypeptide	Position corresponding to position 1486 of isoform A of human Na _v 1.5 alpha subunit polypeptide	Position corresponding to position 1487 of isoform A of human Na _v 1.5 alpha subunit polypeptide
Human Na _v 1.5, isoform b	1484	1485	1486
Human Na _v 1.5, isoform c	1485	1486	1487
Human Na _v 1.5, isoform d	1467	1468	1469

In another aspect, the present invention provides a nucleic acid encoding the above-mentioned mutated Na_v alpha subunit polypeptide.

The invention further provides a variant or fragment of the above-noted polypeptide, the variant or fragment comprising the above-noted mutations at positions corresponding to positions 372, 898, 1419, 1485, 1486, 1487 and 1711 of isoform A of the human Nav1.5 alpha subunit polypeptide noted above, the variant or fragment further having an increased permeability for a group IIA divalent cation (e.g., Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺) and a decreased inactivation rate (relative to a corresponding variant or fragment of a Na_v polypeptide lacking the above-noted mutations). In an embodiment, the above-mentioned variant is a splice variant. Known splice variants of Na_v alpha subunit nucleic acids are described in Chahine *et al.* (*CNS & Neurological Disorders – Drug Targets*, 2008, 7:144-158).

In another aspect, the present invention provides a nucleic acid encoding the above-mentioned mutated Na_v alpha subunit polypeptide, or variant or fragment thereof.

The nucleic acid of the present invention includes those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of any of the Na_v alpha subunit mutant polypeptides described herein. In an embodiment, the above-mentioned nucleic acid comprises the nucleotide sequence of SEQ ID NO: 1, 2, 3 or 4.

Mutagenesis can be performed utilizing any one of several techniques known to those of skill in the art (see for example, Jeff Braman, in *In Vitro* Mutagenesis Protocols, 2nd edition (2002), Humana Press, 304 pages). Moreover, kits for site-directed mutagenesis are commercially available, such as Quikchange™ Site-Directed Mutagenesis Kit from Stratagene, GeneTailor™ Site-Directed Mutagenesis System from Invitrogen, Altered Sites™ *in vitro* Mutagenesis System from Promega.

Various genes and nucleic acid sequences of the invention may be recombinant sequences. Further, polypeptides or proteins of the invention may also be recombinant. The term "recombinant" means that something has been recombined, so that when made in

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reference to a nucleic acid the term refers to a molecule that is comprised of nucleic acid sequences that are joined together or produced by means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein or polypeptide molecule, which is expressed using a recombinant nucleic acid construct created by means of molecular biological techniques. The term "recombinant" when made in reference to genetic composition refers to a gamete or progeny or cell or genome with new combinations of alleles that did not occur in the parental genomes. Recombinant nucleic acid constructs may include a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Referring to a nucleic acid construct as "recombinant" therefore indicates that the nucleic acid molecule has been manipulated using genetic engineering, *i.e.* by human intervention. Recombinant nucleic acid constructs may for example be introduced into a host cell by transformation. Such recombinant nucleic acid constructs may include sequences derived from the same host cell species or from different host cell species, which have been isolated and reintroduced into cells of the host species. Recombinant nucleic acid construct sequences may become integrated into a host cell genome, either as a result of the original transformation of the host cells, or as the result of subsequent recombination and/or repair events.

In accordance with the present invention, an isolated polynucleotide, or an isolated nucleic acid molecule, is a nucleic acid molecule that has been removed from its natural milieu (*i.e.*, that has been subjected to human manipulation), its natural milieu being the genome or chromosome in which the nucleic acid molecule is found in nature. As such, "isolated" does not necessarily reflect the extent to which the nucleic acid molecule has been purified, but indicates that the molecule does not include an entire genome or an entire chromosome in which the nucleic acid molecule is found in nature. An isolated nucleic acid molecule can also include a specified nucleic acid sequence flanked by (*i.e.*, at the 5' and/or the 3' end of the sequence) additional nucleic acids that do not normally flank the specified nucleic acid sequence in nature (*i.e.*, heterologous sequences). Isolated nucleic acid molecules can include DNA, RNA (*e.g.*, mRNA), or derivatives of either DNA or RNA (*e.g.*, cDNA). Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a protein. Preferably, an isolated nucleic acid molecule of the present invention is produced using recombinant DNA technology (*e.g.*, polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis.

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"Homology" and "homologous" refer to sequence similarity between two peptides or two nucleic acid molecules. Homology can be determined by comparing each position in the aligned sequences. A degree of homology between nucleic acid or between amino acid sequences is a function of the number of identical or matching nucleotides or amino acids at positions shared by the sequences. As the term is used herein, a nucleic acid or polypeptide sequence is "homologous" to another sequence if the two sequences are substantially identical and the functional activity of the sequences is conserved (as used herein, the term 'homologous' does not infer evolutionary relatedness). Two nucleic acid or polypeptide sequences are considered "substantially identical" if, when optimally aligned (with gaps permitted), they share at least about 50% sequence similarity or identity and/or if the sequences share defined functional motifs. In alternative embodiments, sequence similarity in optimally aligned substantially identical sequences may be at least 60%, 70%, 75%, 80%, 85%, 90% or 95% with any of the polypeptide or nucleic acid sequences of the invention. As used herein, a given percentage of homology between sequences denotes the degree of sequence identity in optimally aligned sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, though preferably less than about 25 % identity, with any of the polypeptide or nucleic acid sequences of the invention. "Substantially complementary" nucleic acids are nucleic acids in which the complement of one molecule is substantially identical to the other molecule.

Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman, 1981, *Adv. Appl. Math* 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443, the search for similarity method of Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85: 2444, and the computerised implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence identity may also be determined using the BLAST algorithm, described in Altschul *et al.*, 1990, *J. Mol. Biol.* 215: 403-10 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information. The BLAST algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold. Initial neighbourhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to

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zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program may use as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992, *Proc. Natl. Acad. Sci. USA* **89**: 10915-10919) alignments (B) of 50, expectation (E) of 10 (or 1 or 0.1 or 0.01 or 0.001 or 0.0001), M=5, N=4, and a comparison of both strands. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

An alternative indication that two nucleic acid sequences are substantially complementary is that the two sequences hybridize to each other under moderately stringent, or preferably stringent, conditions. Hybridization to filter-bound sequences under moderately stringent conditions may, for example, be performed in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C (see Ausubel, *et al.* (eds), 1989, *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). Alternatively, hybridization to filter-bound sequences under stringent conditions may, for example, be performed in 0.5 M NaHPO₄, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.1 x SSC/0.1% SDS at 68°C (see Ausubel, *et al.* (eds), 1989, *supra*). Hybridization conditions may be modified in accordance with known methods depending on the sequence of interest (see Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology -- Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York). Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH.

In embodiments, the invention further provides polypeptides that are purified, isolated or substantially pure. A compound is "substantially pure" when it is separated from the components that naturally accompany it. Typically, a compound is substantially pure when it is at least 60%, more generally 75% or over 90%, by weight, of the total material in a sample. Thus, for example, a polypeptide that is chemically synthesized or produced by recombinant technology will generally be substantially free from its naturally associated components. A nucleic acid molecule is substantially pure when it is not immediately contiguous with (*i.e.*, covalently linked to) the coding sequences with which it is normally contiguous in the naturally occurring genome of the organism from which the DNA of the invention is derived. A

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substantially pure compound can be obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid molecule encoding a polypeptide; or by chemical synthesis. Purity can be measured using any appropriate method such as column chromatography, gel electrophoresis, HPLC, etc.

5 In another aspect, the present invention provides a vector (e.g., a recombinant vector) comprising the above-mentioned nucleic acid.

The recombinant expression vector of the present invention can be constructed by standard techniques known to one of ordinary skill in the art and found, for example, in Sambrook *et al.* (1989) in *Molecular Cloning: A Laboratory Manual*; and Sambrook and Russell
10 (2001) *Molecular Cloning: A Laboratory Manual* (3rd edition; Cold Spring Harbor Laboratory). A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and can be readily determined by persons skilled in the art. The vectors of the present invention may also contain other sequence elements to facilitate vector propagation. In addition, the vectors of the present invention may comprise a
15 sequence of nucleotides for one or more restriction endonuclease sites. Coding sequences such as for reporter genes are well known to persons skilled in the art. In an embodiment, the vector further comprises one or more gene(s) of interest.

A recombinant expression vector comprising a nucleic acid sequence of the present invention may be introduced into a cell, e.g., a host cell, which may include a living cell
20 capable of expressing the protein coding region from the defined recombinant expression vector. Accordingly, the invention also provides host cells, such as isolated host cells, containing the nucleic acid or recombinant expression vector of the invention. The terms "cells", "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular subject cell, but also to the progeny or potential progeny of such a cell.
25 Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

Vectors can be introduced into cells via conventional transformation or transfection techniques. The terms "transformation" and "transfection" refer to techniques for
30 introducing foreign nucleic acid into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can for example be found in Sambrook *et al.* (*supra*), Sambrook and Russell (*supra*) and other laboratory manuals. Methods for introducing nucleic acids into mammalian cells *in vivo* are also
35 known, and may be used to deliver the vector DNA of the invention to a subject for gene therapy.

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Suitable host cells and methods for recombinant expression of proteins are well known in the art. For example, eukaryotic host cells such as mammalian cells may be used (e.g., rodent cells such as mouse, rat and hamster cell lines, human cell/cell lines). In an embodiment, the above-mentioned cell does not naturally (or endogenously) express a voltage-gated sodium channel. In another embodiment, the above-mentioned cell is a human embryonic kidney cell (e.g., HEK293 cells).

In another aspect, the present invention provides a method for determining whether a test compound modulates the activity of a voltage gated sodium channel, said method comprising

(a) providing a cell expressing the above-mentioned mutated Na_v alpha subunit polypeptide; and
(b) determining the intracellular level of a group IIA divalent cation (e.g., Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺) in said cell in the presence or absence of said test compound, wherein a difference in the intracellular level of said group IIA divalent cation in the presence relative to the absence of said test compound is indicative that said test compound modulates the activity of a voltage gated sodium channel.

In an embodiment, the above-mentioned method comprises providing a source of said group IIA divalent cation in the extracellular medium. Such source of divalent cation may be a solution comprising a chemical compound (e.g., a salt) comprising the divalent cation of interest. For example, a source of calcium (Ca²⁺) may be a solution comprising a suitable amount of calcium chloride (CaCl₂). In an embodiment, the above-mentioned group IIA divalent cation is calcium (Ca²⁺).

Methods and reagents for measuring intracellular ions levels are well known in the art. For example, methods and reagents for detecting intracellular magnesium (Mg²⁺) levels are described in U.S. Patent publications Nos. 20090286275, 20090155837 and 20080293088. Similarly, methods and reagents for measuring intracellular Ca²⁺ levels are well known in the art.

For example, fluorescent indicator compounds suitable for measuring intracellular calcium levels include various calcium indicator dyes (e.g., fluo-3, fura-2, fluo-4, fluo-5, calcium green-1, Oregon green, 488 BAPTA, SNARF-1, and indo-1; see Velicelebi *et al.*, 1999, *Meth. Enzymol.* **294**: 20-47).

Calcium indicator dyes are substances which show a change in a fluorescent characteristic upon binding calcium, e.g., greatly increased intensity of fluorescence and/or a change in fluorescent spectra (*i.e.*, a change in emission or excitation maxima). Fluo-3, fura-2, and indo-1 are commonly used calcium indicator dyes that were designed as structural analogs of the highly selective calcium chelators ethylene glycol-bis(.beta.-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid

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(BAPTA). The fluorescence intensity from fluo-3 increases by more than 100-fold upon binding of calcium. While the unbound dye exhibits very little fluorescence, calcium-bound fluo-3 shows strong fluorescence emission at 526 nm. Fura-2 is an example of a dye that exhibits a change in its fluorescence spectrum upon calcium binding. In the unbound state, fura-2 has an excitation maximum of 362 nm. This excitation maximum shifts to 335 nm upon calcium binding, although there is no change in emission maximum. Binding of calcium to fura-2 can be monitored by excitation at the two excitation maxima and determining the ratio of the amount of fluorescence emission following excitation at 362 nm compared to the amount of fluorescence emission following excitation at 335 nm. A smaller ratio (i.e., less emission following excitation at 362 nm) indicates that more fura-2 is bound to calcium, and thus a higher internal calcium concentration in the cell.

The use of calcium indicator dyes entails loading cells with the dye, a process which can be accomplished by exposing cells to the membrane-permeable acetoxymethyl esters of the dyes. Once inside the plasma membrane of the cells, intracellular esterases cleave the esters, exposing negative charges in the free dyes. This prevents the free dyes from crossing the plasma membrane and thus leaves the free dyes trapped in the cells. Measurements of fluorescence from the dyes are then made, the cells are treated in such a way that the internal calcium concentration is changed (e.g., by exposing cells to an activator or inhibitor of a voltage-gated ion channel), and fluorescence measurements are again taken.

Fluorescence from the indicator dyes can be measured with a luminometer or a fluorescence imager. One preferred detection instrument is the Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, Calif.). The FLIPR is well suited to high throughput screening using the methods of the present invention as it incorporates integrated liquid handling capable of simultaneously pipetting to 96 or 384 wells of a microtiter plate and rapid kinetic detection using a argon laser coupled to a charge-coupled device imaging camera.

One skilled in the art would be able to optimize experimental parameters (cell number, dye concentration, dye loading time, temperature of incubations, cell washing conditions, and instrument settings, etc.) by routine experimentation depending on the particular relevant experimental variables (e.g., type of cell used, identity of dye used). Several examples of experimental protocols that can be used are described in Velicelebi *et al.*, 1999, *supra*.

In particular embodiments, the change in fluorescent characteristic is an increase in intensity of a fluorescence emission maximum. In other embodiments, the change in fluorescent characteristic is a shift in the wavelength of an absorption maximum. It is understood in the art that absorption of radiation results in the excitation of fluorescent molecule, and the terms "absorption" and "excitation", for example in respect of spectra, wavelengths, and maxima, are used interchangeably herein.

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In an embodiment, the cells naturally express the mutated voltage-gated ion channel of interest. In another embodiment, the cells have been transfected (or transformed) with an expression vector that encode the voltage-gated ion channel of interest so that the cells recombinantly express the voltage-gated ion channel of interest. Transfection is meant to include any method known in the art for introducing nucleic acids such as expression vectors into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct, and electroporation.

Another way to measure ion flow indirectly is to monitor changes in transcription that result from the activity of voltage-gated ion channels by the use of transcription based assays. Transcription-based assays involve the use of a reporter gene whose transcription is driven by an inducible promoter whose activity is regulated by a particular intracellular event such as, e.g., changes in intracellular calcium levels, that are caused by the activity of a voltage-gated ion channel. Transcription-based assays are reviewed in Rutter *et al.*, 1998, *Chemistry & Biology* 5: R285-R290. Transcription-based assays of the present invention rely on the expression of reporter genes whose transcription is activated or repressed as a result of intracellular events that are caused by the interaction of a activator or inhibitor with a voltage-gated ion channel.

In an embodiment, the intracellular calcium level is determined by determining the conformational change of a calcium-binding polypeptide.

As used herein, the term "calcium-binding polypeptide" refers to a polypeptide that binds one or more calcium ions and which undergoes a conformational change upon calcium binding. Examples of calcium-binding polypeptides include, for example, Calmodulin (CaM) as well as proteins known as Calmodulin-like proteins such as aequorin, Calcium-binding protein (CABP), Calcineurin B subunit isoform 1, Calmodulin-related protein NB-1 (CLP), Calcium vector protein (CAVP), Guanylyl cyclase activating protein 3 (GCAP 3), Calcium and integrin-binding protein 1 (Calmyrin; KIP; CIB), Myosin-2 light chain, Myosin essential light chain striated adductor muscle (E-LC), Myosin regulatory light chain striated adductor muscle (R-LC), Myosin regulatory light chain cdc4, Neurocalcin delta, Neuronal calcium sensor 1 (Frequenin), Sarcoplasmic calcium-binding protein (SCP), Troponin C, and any calcium-binding fragment of the above-mentioned proteins. Therefore, any assay (e.g., fluorescent-based, enzymatic-based) which permits to measure the level/extent of conformational change of a calcium-binding polypeptide may be used in the method of the present invention.

For example, the intracellular calcium level may be measured using the aequorin system. The aequorin system makes use of the protein apoaequorin, which binds to the lipophilic chromophore coelenterazine forming a combination of apoaequorin and coelenterazine that is known as aequorin. Apoaequorin has three calcium binding sites and,

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upon calcium binding, the apoaequorin portion of aequorin changes its conformation. This change in conformation causes coelenterazine to be oxidized into coelenteramide, CO₂, and a photon of blue light (466 nm). This photon can be detected with suitable instrumentation. Reagents to analyze intracellular calcium levels based on the aequorin system are commercially available. For example, recombinant aequorin (AquaLite®, Cat. No. A-6785) as well as coelenterazine and derivatives thereof (e.g., Cat. Nos. C-2944, C-14261, C-14261, C-6779 and C-6780) may be purchased at Invitrogen/Molecular Probes.

In an embodiment, the above-mentioned calcium-binding polypeptide is calmodulin (CaM) or a calcium-binding fragment thereof. Calmodulin is an intracellular calcium receptor found ubiquitously in eukaryotes. It is capable of regulating biological activities of many cellular proteins and transmembrane ion transporters mainly in a Ca²⁺-dependent manner. When the intracellular calcium level rises to a certain level, four Ca²⁺ ions bind to calmodulin, and the Ca²⁺-calmodulin complex binds the target proteins (e.g., skeletal muscle myosin light chain kinase, smooth muscle myosin light chain kinase, calmodulin-dependent kinase II), initiating various signalling cascades. Therefore, intracellular calcium levels may be measured, for example, by assessing the level of binding of a target protein to CaM, or by assessing the activation of a signalling cascade which depends on the CaM activation. For example, the binding of calcium ions to CaM allows CaM to bind to cyclic nucleotide phosphodiesterases and to adenylyl cyclase with subsequent activation, thereby increasing cyclic AMP and cyclic GMP levels. Therefore, an increase in intracellular calcium levels may be measured indirectly by determining cyclic AMP and/or cyclic GMP levels in a cell.

As noted above, calcium-binding polypeptides bind one or more calcium ions and generally undergo conformational change upon calcium binding. This conformational change allows, in turn, the interaction with other proteins in a calcium-dependent manner. As such, a modulation in intracellular Ca²⁺ levels may be measured by determining the extent of binding between a calcium-binding polypeptide and a ligand or protein that binds to the calcium-binding polypeptide in a calcium-dependent manner (*i.e.*, upon calcium-induced conformational change in the calcium-binding polypeptide). Therefore, any combination of a calcium-binding polypeptide with a ligand/polypeptide that binds to the calcium-binding polypeptide in a calcium-dependent manner may be used in the method of the present invention.

In an embodiment, the above-mentioned calcium-binding polypeptide is calmodulin (CaM) or a calcium-binding fragment thereof, and the intracellular calcium levels is determined by assessing the binding of a calcium-dependent calmodulin-binding protein to CaM (or to a calcium-binding fragment thereof). Examples of calcium-dependent calmodulin-binding proteins include, for example, peripheral plasma membrane protein CASK, Elongation factor 2 kinase, Calcium/calmodulin-dependent protein kinase type I, Calcium/calmodulin-dependent

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protein kinase type IV catalytic chain, Calcium/calmodulin-dependent protein kinase type II alpha chain, Myosin light chain kinase/smooth muscle and non-muscle isozymes, Phosphorylase B kinase alpha regulatory chain/skeletal muscle isoform, Calcineurin B subunit isoform 1, Ca^{2+} /calmodulin-dependent protein kinase phosphatase, Serine/threonine protein phosphatase 2B catalytic subunit alpha isoform, Cysteinyl leukotriene receptor 2, Adenylate cyclase type I, brain Nitric-oxide synthase, Inositol-trisphosphate 3-kinase A, Caldesmon, Dystrophin, Myristoylated alanine-rich C-kinase substrate, Alpha-1-syntrophin, erythrocyte Spectrin alpha chain and brain Spectrin alpha chain.

In an embodiment, the above-mentioned calcium-dependent calmodulin binding protein is myosin light chain kinase or a calmodulin-binding domain thereof. In a further embodiment, the above-mentioned calmodulin-binding domain of a myosin light chain kinase comprises a domain corresponding to residues 577 to 602 of rabbit skeletal muscle myosin light chain kinase (generally referred to as M13, KRRWKKNFIAVSAANRFKKISSSGAL, SEQ ID NO: 14). In a further embodiment, the above-mentioned calmodulin-binding domain of a myosin light chain kinase comprises the amino acid sequence of SEQ ID NO: 14.

In another embodiment, the above-mentioned cell expressing a mutated Na_v alpha subunit polypeptide further expresses a first and second marker polypeptide, wherein

(i) said first marker polypeptide comprises:

(a) a first domain comprising a first agent having an energy emission spectrum;

(b) a second domain linked to said first domain and comprising a calcium-binding polypeptide;

(ii) said second marker polypeptide comprises:

(a) a first domain comprising a polypeptide binding in a calcium-dependent manner to said calcium-binding polypeptide; and

(b) a second domain linked to said first domain of said second marker polypeptide and comprising a second agent having an absorption spectrum which overlaps with the emission spectrum of said first agent;

wherein the intracellular calcium level is determined by measuring the intensity of the emission emitted by said second agent.

In another embodiment, the above-mentioned cell expressing a mutated Na_v alpha subunit polypeptide further expresses a first and second fluorescent polypeptide, wherein

(i) said first fluorescent polypeptide comprises:

(a) a first domain comprising a first fluorescent agent having an emission spectrum;

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- (b) a second domain linked to said first domain and comprising a calcium-binding polypeptide;
- (ii) said second fluorescent polypeptide comprises:
- (a) a first domain comprising a polypeptide binding in a calcium-dependent manner to said calcium-binding polypeptide; and
- (b) a second domain linked to said first domain of said second polypeptide and comprising a second fluorescent agent having an absorption spectrum which overlaps with the emission spectrum of said first fluorescent agent;

wherein the intracellular calcium level is determined by measuring the intensity of the fluorescence emitted by said second fluorescent agent.

As noted above, any combination of a calcium-binding polypeptide (second domain of the first chimeric polypeptide) with a polypeptide that binds to the calcium-binding polypeptide in a calcium-dependent manner (first domain of the second chimeric polypeptide) may be used in the method of the present invention.

In an embodiment, the calcium-binding polypeptide is calmodulin or a calcium binding fragment thereof, and the first domain of the second chimeric polypeptide comprises a calcium-dependent calmodulin-binding polypeptide, such as those described above.

The first and second fluorescent agents are selected according to their characteristic absorption and emission spectra. The excitation energy of the first fluorescent agent (the donor) is transferred to the second fluorescent agent (the acceptor) by dipolar interactions, without donor fluorescence emission. This is generally referred to as Fluorescence Resonance Energy Transfer (FRET). The donor emission and acceptor absorption spectra should overlap for FRET to occur, and therefore any combination of fluorescent agents having overlapping emission/absorption spectra may be used in the method of the present invention. Combinations of fluorescent agents that are suitable for FRET-based experiments are well-known in the art. In embodiments, the configuration of the above-noted first and second polypeptides may be modified such that the above-noted agent having an emission spectrum and agent having an absorption spectrum are reversed, i.e., such that the first (fluorescent) polypeptide comprises a first domain comprising a first (fluorescent) agent having an absorption spectrum, and the second (fluorescent) polypeptide comprises a second domain comprising a second (fluorescent) agent having an emission spectrum which overlaps with the absorption spectrum of the first (fluorescent) agent. "Overlap" as used in the context of fluorescent spectra refers to the ability of the emitted light from a fluorophore to be of a wavelength capable of excitation of another fluorophore.

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In an embodiment, the above-mentioned fluorescent agent is a polypeptide comprising a fluorescent protein, such as the Green Fluorescent Protein (GFP) as well as all derivatives thereof such as Blue Fluorescent Protein (BFP), Red Fluorescent Protein (RFP), Yellow Fluorescent Protein (YFP), Cyan Fluorescent Protein (CFP). Fluorescent polypeptide pairs that are suitable for FRET-based experiments include, for example, CFP and YFP (or a modified YFP called Venus) as well as BFP and GFP.

In an embodiment, the above-mentioned first fluorescent agent is a cyan fluorescent protein (CFP) and said second fluorescent agent is a yellow fluorescent polypeptide (YFP).

In an embodiment, the above-mentioned first fluorescent agent is a yellow fluorescent polypeptide (YFP) and said second fluorescent agent is a cyan fluorescent protein (CFP).

In an embodiment, the above-mentioned CFP comprises the following mutations relative to wild-type GFP: F64L / S65T / Y66W / N146I / M153T / V163A / N164H. In an embodiment, the above-mentioned YFP comprises the following mutations relative to wild-type GFP: S65G / S72A / T203Y. In an embodiment, the above-mentioned YFP comprises the following mutations relative to wild-type GFP: S65G / V68L / Q69K / S72A / T203Y (see, for example, Miyawaki *et al.*, *Proc Natl Acad Sci USA*. 1999, **96**: 2135–2140).

In cases where the fluorescent agent is a polypeptide comprising a fluorescent protein, the first and/or second fluorescent polypeptide(s) may be recombinantly expressed as a chimeric polypeptide. For example, a cell may comprise a nucleic acid encoding a fluorescent protein may be linked to a nucleic acid encoding a calcium-binding polypeptide (or a polypeptide binding in a calcium-dependent manner to a calcium-binding polypeptide), for expression of a chimeric polypeptide comprising the fluorescent protein and the calcium-binding polypeptide (or the polypeptide binding in a calcium-dependent manner to a calcium-binding polypeptide) covalently linked to each other.

In another embodiment, the above-mentioned first and second fluorescent polypeptides are recombinantly expressed as a chimeric polypeptide. For example, the second domain of the first fluorescent polypeptide may be linked to the first domain of the second fluorescent polypeptide, and thus the four domains are linked to each other and expressed as a single chimeric polypeptide. An example of such a chimeric system is the Cameleon calcium sensor system.

The Cameleon molecule comprises four domains. The Cameleon is a fusion product between two fluorescent proteins (having differing excitation and emission characteristics), calmodulin (CaM), and the calmodulin-binding domain of rabbit myosin light chain kinase (M13). Calmodulin is capable of binding with free calcium ions and the M13 chain

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can bind with calmodulin after it has bound the calcium ions. The nucleic acids encoding these four proteins are joined linearly, and the fusion nucleic acid may be expressed in a variety of cells. Fluorescent proteins are located at both ends of the chimera and thus stand away from each other in the absence of calcium ion. But in the presence of calcium ions, the activated
5 CaM linker wraps around the M13 protein. The tertiary structure of the Cameleon chimera is then altered to bring the two fluorescent protein moieties closer to each other and thus increases the efficiency of energy transfer (e.g., FRET).

The binding of calcium by the calmodulin moiety of Cameleon produces a conformational change of the entire molecule, which positions the two fluorescent proteins into
10 close spatial proximity. In this conformation, dipolar energy transfer by the excited first fluorescent protein (e.g., CFP) protein stimulates the second fluorescent protein (e.g., YFP, or a modified YFP called Venus (Nagai T. *et al.*, *Nat. Biotechnol.* 2002, **20**: 87-90)) to produce secondary fluorescence having a wavelength that is different than the wavelength of the fluorescence emitted by the first fluorescent protein. In such a system, intracellular calcium ion
15 concentration can be determined by fluorescence ratio imaging as the ratio of the changes in two types of fluorescence (*i.e.*, the fluorescence emitted by the first fluorescent protein versus that emitted by the second fluorescent protein). An increase in the fluorescence emitted by the second fluorescent protein versus that emitted by the first fluorescent protein is indicative that FRET occurs, and thus that the level of intracellular calcium is increased.

The above-mentioned methods may be employed either with a single test
20 compound or a plurality or library (e.g., a combinatorial library) of test compounds. In the latter case, synergistic effects provided by combinations of compounds may also be identified and characterized. The above-mentioned compounds may be used for prevention and/or treatment of diseases associated with abnormal activity of a voltage-gated sodium channel (or in which
25 modulation of voltage-gated sodium channel activity would be beneficial), or may be used as lead compounds for the development and testing of additional compounds having improved specificity, efficacy and/or pharmacological (e.g., pharmacokinetic) properties. In an embodiment the compound may be a prodrug which is altered into its active form at the appropriate site of action, (e.g., a cell, tissue or organ in which abnormal activity of a voltage-
30 gated sodium channel is observed, such as a central nervous system (CNS) cell, tissue or organ). In certain embodiments, one or a plurality of the steps of the screening/testing methods of the invention may be automated. Such methods may be performed in an array format.

There are several diseases/conditions associated with abnormal activity of a voltage-gated sodium channel (general referred to as channelopathies). For example, mutations
35 in the gene encoding Na_v1.5 cause various diseases/conditions such as inherited long QT syndrome type 3, Brugada syndrome, conduction disease, sinus node dysfunction, and atrial

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standstill, which lead to an increased risk of ventricular arrhythmias (Remme CA *et al.*, *Trends Cardiovasc Med.* 2008, **18**(3): 78-87).

As such, the screening methods of the present invention may be useful for identifying compounds that may be used for the prevention and/or treatment of one or more of the above-mentioned diseases/conditions, or any other disease/condition associated with aberrant voltage-gated sodium channel function. Accordingly, in another aspect, the present invention provides a compound identified by the above-mentioned method, as well as the use of such compounds for the prevention and/or treatment of disease/condition associated with aberrant voltage-gated sodium channel function.

In an embodiment, the above-mentioned chimeric polypeptide comprises the amino acid sequence of a Cameleon polypeptide.

In another aspect, the present invention provides a cell comprising (a) the above-mentioned mutated Na_v alpha subunit polypeptide and (b) the above-mentioned first and second polypeptides.

In another aspect, the present invention provides a kit comprising the above-mentioned cell and a container. In another aspect, the present invention provides a kit comprising the above-mentioned vector and a container. Such kit may further comprise, for example, instructions for determining whether a test compound modulates the activity of a voltage gated sodium channel, control samples, reagents useful for performing the methods (e.g., buffers, enzymes, transfection reagents, detection reagents), host cells, etc.

MODE(S) FOR CARRYING OUT THE INVENTION

The present invention is illustrated in further details by the following non-limiting examples.

Example 1: Materials and methods

Site-directed mutagenesis. Site-directed mutations to wild-type human Na_v1.5 was made using the QuickChange™ Site-directed Mutagenesis Kit according to the manufacturer instructions (Stratagene Inc., La Jolla, CA). Base substitutions were confirmed by automatic DNA sequencing at the CHUL Research Centre DNA sequencing facility (Québec, Québec).

Transient expression of Na channels in HEK293 cells. A standard calcium phosphate precipitation procedure was used to transfect the cDNA of the wild-type and mutant Na_v1.5 channel (cloned into the pCDNA3neo vector) into HEK293 cells. For transfection, Na_v1.5 DNA (10 µg) was mixed with 10 µg of EBO plasmid encoding for the CD8 antigen and 0.5 ml of 250 mM CaCl₂. This mixture was then slowly added to 0.5 ml of 2x HeBS solution comprising (in mM): 275 NaCl, 40 Hepes, 12 dextrose, 10 KCl, 1.4 Na₂HPO₄, pH 7.5. This mixture was

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incubated 20 minutes at room temperature then slowly added to a 100 mm culture dish of 50% confluent HEK293 cells bathed in 10 ml of DMEM (Gibco) enriched with 10% fetal bovine serum and 1% penicillin-streptomycin. After 12 hours of incubation the cells were washed and replated on 35 mm culture dishes. Currents were recovered within 12-24 hours of plating.

5 *Establishment of reporter cell lines.* The reporter cell lines described herein are derived from the HEK293 cell line, which, in turn, is derived from human embryonic kidney. HEK293 cells are of human origin, display high cell division efficiency and do not express significant endogenous sodium channels. To generate a $\text{Na}_v1.5^{\text{Mut}}$ /Cameleon reporter cell line, the first step was to establish a HEK293 cell line which expresses the Cameleon calcium
10 biosensor alone. HEK293 cells were transfected with a vector (pIRESHyg3, Clontech, Cat. No. 631620) encoding the Cameleon construct (as described in Evanko and Haydon, *Cell Calcium* (2005) **37**: 341-348). This cell line that express the Cameleon alone was then stably transfected with the pcDNA4neo- $\text{Na}_v1.5^{\text{Mut}}$ expression vector (see Fig. 7A for the sequence of $\text{Na}_v1.5^{\text{Mut}}$). After two weeks of selection with hygromycin to eliminate the cells that do not express the
15 vector, the resulting cell colonies were transferred to a 96, 48, 24, 12 and 6 wells culture plates for amplification. The resulting cell lines were then assessed in patch-clamp for their capacity to produce robust current amplitude.

Whole-cell patch clamp recording. Whole-cell Na current recordings were obtained using an Axopatch™ 200A patch clamp amplifier equipped with a DigiData™ 1200
20 interface (Axon Instruments). Voltage pulses were generated and data collected using pClamp™ (Axon Instruments). Patch pipettes were fashioned from Corning™ 8161 borosilicate glass (Dow Corning), have resistances of 0.5-2 MΩ, and are sylgard coated to reduce capacitance transients. Whole-cell recordings are leak corrected using P/4 subtraction. Typical extracellular solution contains (in mM): 140 NaCl, 2 KCl, 1.5 CaCl_2 , 1 MgCl_2 and 10 HEPES pH
25 7.4 with NaOH. Internal solution contains (in mM): 105 CsF, 35 NaCl, 5 EGTA and 10 HEPES pH 7.4 with CsOH. Cells expressing the target cDNA were identified using beads coated with an antibody against CD8 (Dynabeads, Dynal Corp.).

Development of Cameleon assay. Cameleon is a fluorescent biosynthetic Ca^{2+} indicator constructed by inserting a Ca^{2+} sensor (Calmodulin and M13, a calmodulin-binding
30 protein) between two mutated forms of green fluorescent protein (GFP): the energy acceptor Venus, a derivative of yellow fluorescent protein (YFP), and the energy donor, cyan fluorescent protein (CFP) (Evanko DS and Haydon PG, *Cell calcium* 2005, **37**: 341-348). Cameleon fluorescence is affected by differences in the concentration of Ca^{2+} that alter the amount of fluorescence resonance energy transfer (FRET) between CFP and Venus. This process is
35 influenced by Ca^{2+} -induced change in the conformation of calmoduline-M13, which, consequently, alters the relative angular displacement between the two mutant GFPs, bringing

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them closer together, for example, following an increase in $[Ca^{2+}]$. The increase in FRET is directly correlated to $[Ca^{2+}]$ increase. The fluorescence intensity was measured using a Packard Fusion™ Universal Microplate Analyzer. 75,000 cells per well were analyzed in a bath solution containing 140 mM NaCl, 2 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Na-HEPES. For calcium measurements, the bath solution contained: 140mM NaCl, 2mM KCl, 25 mM $CaCl_2$, 10 mM glucose and 10mM Na-HEPES. The pH of the bath solutions was adjusted to pH 7.4 using 1N NaOH.

Example 2: Effect of the mutations on calcium permeability

The results presented at Fig. 2 clearly show that the mutated $Na_v1.5$ sodium channel is still permeable to Na^+ ions (Fig. 2A), but is also permeable to Ca^{2+} ions (Fig. 2B), which is the effect of the mutation in the "DEKA" motif. Fig. 2 also shows that there is a persistent current in both situations (Fig. 2A and B), which is likely due to a decreased/slower channel deactivation caused by the mutation in the "IFM" motif.

Example 3: HEK293 cells stably expressing the Cameleon biosensor

A HEK293 cell line stably expressing the Cameleon biosensor alone was treated with ionomycin (10 μ M), a calcium ionophore, to raise the intracellular level of Ca^{2+} , and the FRET ratio was analysed by confocal microscopy. As shown in Fig. 3B, the Cameleon biosensor can be stably expressed in HEK293 cell and permits to detect intracellular Ca^{2+} fluctuations, as indicated by the increase in the Venus/CFP ratio (i.e. increase in FRET) following addition of ionomycin.

Example 4: HEK293 cells stably expressing the mutated $Na_v1.5$ polypeptide and the Cameleon biosensor (VC/ $Na_v1.5$)

The Z'-factor (a measure of the quality or power of a high-throughput screening (HTS) assay) was analyzed in a fluorescence-microplate-based assay. Z'-factor values of 0.72 and 0.69 were obtained for the VC/ $Na_v1.5$ #12 and #14 cell lines, respectively (Fig. 4A). For a HTS assay, a Z'-factor between 0.5 and 1 is considered "excellent" (Zhang JH *et al.*, *J Biomol Screen* 1999, 4(2): 67-73). The effect of veratridine, a sodium channel agonist, was also assessed and the results showed that VC/ $Na_v1.5$ #12 and #14 cell lines respond to veratridine as well as ionomycin treatment, whereas VC #7, the cell line that does not express a sodium channel, respond only to ionomycin (Fig. 4B). These results indicate that the $Na_v1.5^{Mut}$ /Cameleon cells express a functional sodium channel that is permeable to calcium ions, and that the Cameleon biosensor is able to detect the modulation of Ca^{2+} caused by activation of the $Na_v1.5^{Mut}$ channel.

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Fig. 5 shows that $\text{Na}_v1.5^{\text{Mut}}$ /Cameleon cell lines respond to different concentrations of extracellular calcium. The intensity of the FRET ratio signals detected correlates with calcium concentrations, as shown by the correlation coefficient of 0.996 in the $\text{Na}_v1.5^{\text{Mut}}$ /Cameleon cell line, indicative of a strong standard dose-response relationship. Such a strong dose-response relationship was not observed with the control Cameleon cell line ($R^2 = 0.657$).

Dose-response experiments in the presence of known sodium channel blockers (quinidine, sertraline and paroxetine) were performed. As shown in Fig. 6, addition of increasing doses of quinidine (Fig. 6A), sertraline (Fig. 6B) or paroxetine (Fig. 6C) results in a decrease in the Delta FRET ratio signal as compared to control cells (cells receiving buffer only), with a Z' value greater than 0.65 over the 96-well plate. These results demonstrate that drug-induced inhibition of sodium channels may be detected using a cell expressing a mutated Ca^{2+} permeable sodium channel and a Cameleon biosensor.

Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to". The singular forms "a", "an" and "the" include corresponding plural references unless the context clearly dictates otherwise.

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WHAT IS CLAIMED IS:

1. A mutated voltage gated sodium channel (Na_v) alpha subunit polypeptide comprising a first mutation that increases the permeability of the channel for a group IIA divalent cation and a second mutation that decreases the inactivation rate of the channel.
- 5 2. The mutated Na_v alpha subunit polypeptide of claim 1, wherein said first mutation is a mutation in a domain connecting a S5 and a S6 α-helical transmembrane-spanning segment.
3. The mutated Na_v alpha subunit polypeptide of claim 2, wherein said first mutation is:
 - (i) a mutation at a residue corresponding to residue 372 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
 - 10 (ii) a mutation at a residue corresponding to residue 898 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
 - (iii) a mutation at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
 - (iv) a mutation at a residue corresponding to residue 1711 in the amino acid
 - 15 sequence of isoform A of human Na_v1.5 alpha subunit; or
 - (v) any combination of (i) to (iv).
4. The mutated Na_v alpha subunit polypeptide of claim 3, wherein said first mutation is a mutation at a residue corresponding to residue 1419 in the amino acid sequence of Na_v1.5 alpha subunit.
- 20 5. The mutated Na_v alpha subunit polypeptide of claim 4, wherein said mutation at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit is a substitution to an alanine residue.
6. The mutated Na_v alpha subunit polypeptide of any one of claims 1 to 5, wherein said second mutation is a mutation in an intracellular region connecting the S6 α-helical
- 25 transmembrane-spanning segment of Domain III and the S1 α-helical transmembrane-spanning segment of Domain IV.
7. The mutated Na_v alpha subunit polypeptide of claim 6, wherein said second mutation is:
 - (i) a mutation at a residue corresponding to residue 1485 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;
 - 30 (ii) a mutation at a residue corresponding to residue 1486 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit;

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- (iii) a mutation at a residue corresponding to residue 1487 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit; or
- (iv) any combination of (i) to (iii).

8. The mutated Na_v alpha subunit polypeptide of claim 7, wherein said second mutation is
5 a mutation at a residue corresponding to residue 1486 in the amino acid sequence of Na_v1.5 alpha subunit.

9. The mutated Na_v alpha subunit polypeptide of claim 8, wherein said mutation at a residue corresponding to residue 1486 in the amino acid sequence of isoform A of human Na_v1.5 alpha subunit is a substitution to a glutamine residue.

10 10. The mutated Na_v alpha subunit polypeptide of any one of claims 1 to 9, wherein said first mutation is at a residue corresponding to residue 1419 in the amino acid sequence of isoform A of human Nav1.5 alpha subunit, and said second mutation is at a residue corresponding to residue 1486 in the amino acid sequence of isoform A of human Nav1.5 alpha subunit.

11. The mutated Na_v alpha subunit polypeptide of any one of claims 1 to 10, wherein said
15 mutated Na_v alpha subunit polypeptide is a mutated human Na_v1.5 alpha subunit polypeptide.

12. The mutated Na_v alpha subunit polypeptide of claim 11, comprising the amino acid sequence of SEQ ID NO: 5.

13. The mutated Na_v alpha subunit polypeptide of any one of claims 1 to 12, wherein said group IIA divalent cation is calcium (Ca²⁺).

20 14. An isolated nucleic acid encoding the mutated Na_v alpha subunit polypeptide of any one of claims 1 to 13.

15. The isolated nucleic acid of claim 14, comprising the nucleotide sequence of SEQ ID NO: 1, 2, 3 or 4.

16. A vector comprising the nucleic acid of claim 14 or 15.

25 17. A cell comprising the nucleic acid of claim 14 or 15, or the vector of claim 16.

18. A method for determining whether a test compound modulates the activity of a voltage gated sodium channel, said method comprising

- (a) providing a cell expressing the mutated Na_v alpha subunit polypeptide of any one of claims 1 to 13; and

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(b) determining the intracellular level of a group IIA divalent cation in said cell in the presence or absence of said test compound,

wherein a difference in the intracellular level of said group IIA divalent cation in the presence relative to the absence of said test compound is indicative that said test compound modulates the activity of a voltage gated sodium channel.

19. The method of claim 18, further comprising providing a source of said group IIA divalent cation in the extracellular medium.

20. The method of claim 18 or 19, wherein said group IIA divalent cation is calcium (Ca^{2+}).

21. The method of claim 20, wherein the intracellular calcium level is determined by determining the conformational change of a calcium-binding polypeptide.

22. The method of claim 21, wherein said calcium-binding polypeptide is calmodulin (CaM) or a calcium-binding fragment thereof.

23. The method of any one of claims 17 to 21, wherein said cell further expresses a first and second chimeric polypeptide, wherein

(i) said first chimeric polypeptide comprises:

(a) a first domain comprising a first fluorescent agent having an emission spectra;

(b) a second domain linked to said first domain and comprising a calcium-binding polypeptide;

(ii) said second chimeric polypeptide comprises:

(a) a first domain comprising a polypeptide which binds in a calcium-dependent manner to said calcium-binding polypeptide; and

(b) a second domain linked to said first domain of said second chimeric polypeptide and comprising a second fluorescent agent having an absorption spectra which overlaps with the emission spectra of said first fluorescent agent;

wherein the intracellular calcium level is determined by measuring the intensity of the fluorescence emitted by said second fluorescent agent.

24. The method of any one of claims 17 to 21, wherein said cell further expresses a first and second chimeric polypeptide, wherein

(i) said first chimeric polypeptide comprises:

(a) a first domain comprising a first fluorescent agent having an absorption spectra;

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(b) a second domain linked to said first domain and comprising a calcium-binding polypeptide;

(ii) said second chimeric polypeptide comprises:

5 (a) a first domain comprising a polypeptide which binds in a calcium-dependent manner to said calcium-binding polypeptide; and

(b) a second domain linked to said first domain of said second chimeric polypeptide and comprising a second fluorescent agent having an emission spectra which overlaps with the absorption spectra of said first fluorescent agent; wherein the intracellular calcium level is determined by measuring the intensity of the
10 fluorescence emitted by said first fluorescent agent.

25. The method of claim 23 or 24, wherein said calcium-binding polypeptide is calmodulin or a calcium-binding fragment thereof.

26. The method of claim 25, wherein said polypeptide binding in a calcium-dependent manner to said calcium-binding polypeptide is myosin light chain kinase or a calmodulin-binding
15 domain thereof.

27. The method of claim 26, wherein said calmodulin-binding domain of a myosin light chain kinase comprises a domain corresponding to residues 577 to 602 of rabbit skeletal muscle myosin light chain kinase (M13).

28. The method of claim 27, wherein said calmodulin-binding domain of a myosin light chain
20 kinase comprises the amino acid sequence of SEQ ID NO: 14.

29. The method of claim 23, wherein said first fluorescent agent is a cyan fluorescent protein (CFP) and said second fluorescent agent is a yellow fluorescent polypeptide (YFP).

30. The method of claim 24, wherein said first fluorescent agent is a yellow fluorescent polypeptide (YFP) and said second fluorescent agent is a cyan fluorescent protein (CFP).

25 31. The method of any one of claims 23 to 30, wherein said second domain of said first chimeric polypeptide is linked to said first domain of said second chimeric polypeptide.

32. The method of claim 31, wherein said first and second chimeric polypeptides are recombinantly expressed as a single chimeric polypeptide.

33. The method of claim 32, wherein said single chimeric polypeptide comprises an amino
30 acid sequence of a Chameleon polypeptide.

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34. The method of any one of claims 18 to 33, wherein said mutated Na_v alpha subunit polypeptide is the mutated Na_v alpha subunit polypeptide of claim 10.

35. The cell of claim 17, further comprising a second nucleic encoding the first and second chimeric polypeptides defined in any one of claims 23 to 34.

5 36. A kit comprising

- (a) (i) the cell of claim 17 or 35;
- (ii) the vector of claim 16;
- (iii) both (i) and (ii); and
- (b) a container.

10 37. The kit of claim 36, further comprising instructions setting forth the method of any one of claims 18 to 34.

38. The kit of claim 36 or 37, wherein said vector further comprises the second nucleic acid as defined in claim 35.

15 39. The kit of claim 36 or 37, further comprising a second vector comprising the second nucleic acid as defined in claim 35, and a container.

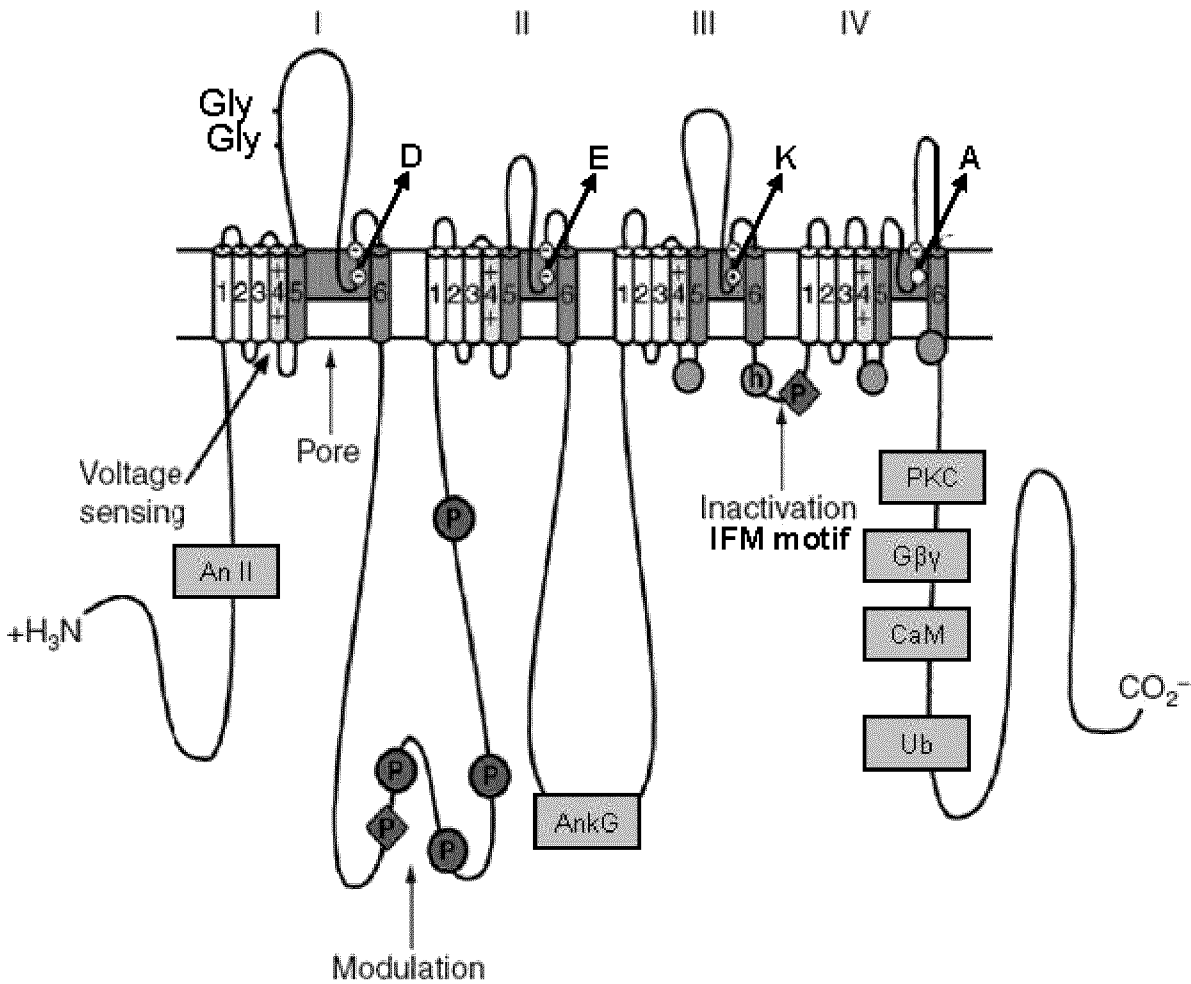


Fig. 1

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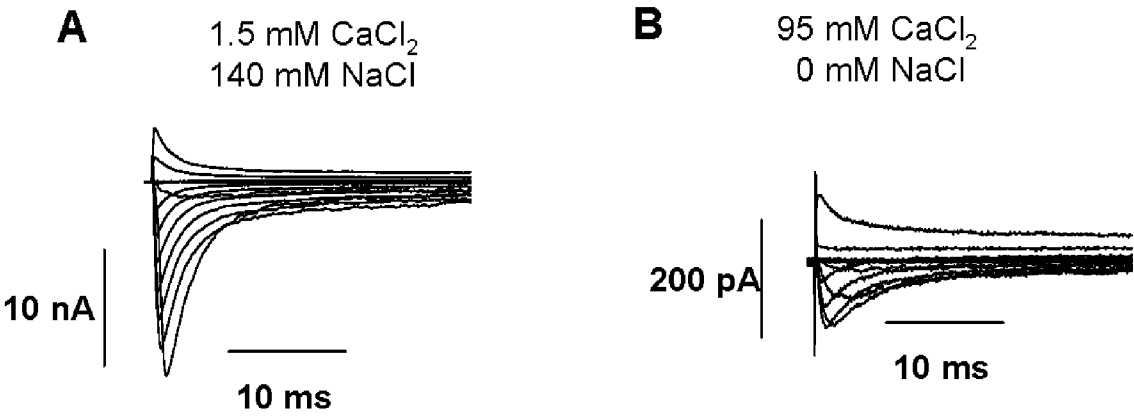


Fig. 2

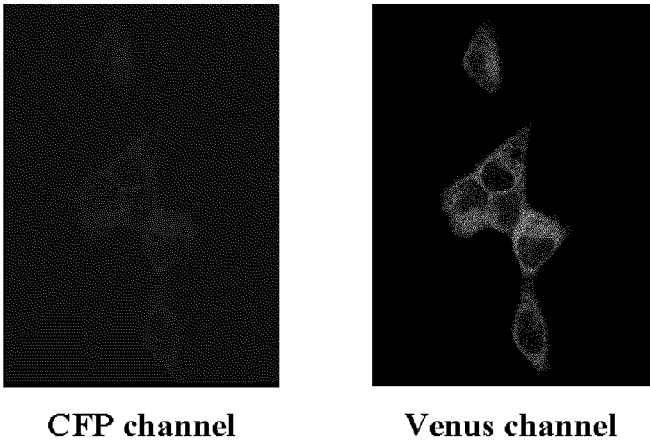


Fig. 3A

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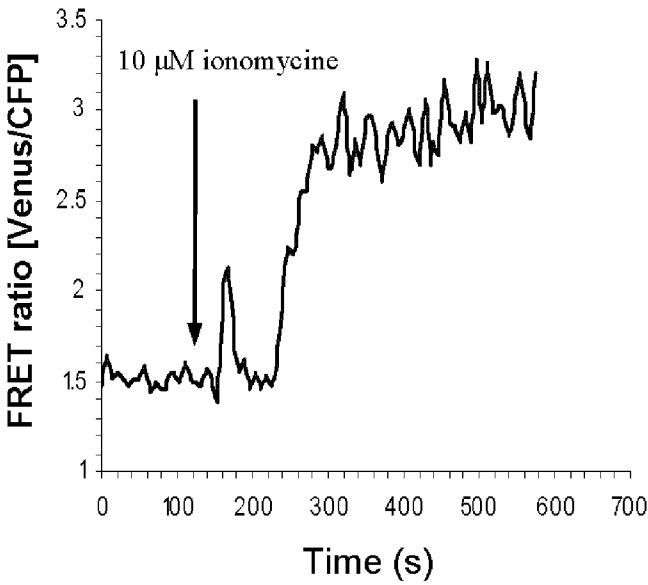


Fig. 3B

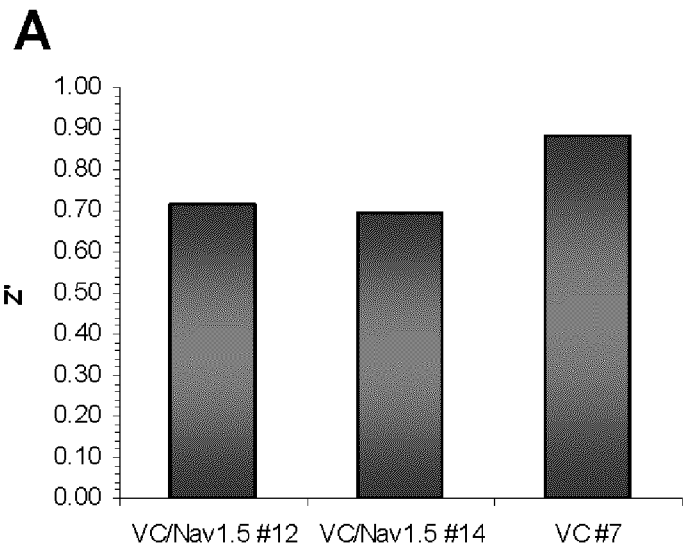


Fig. 4A

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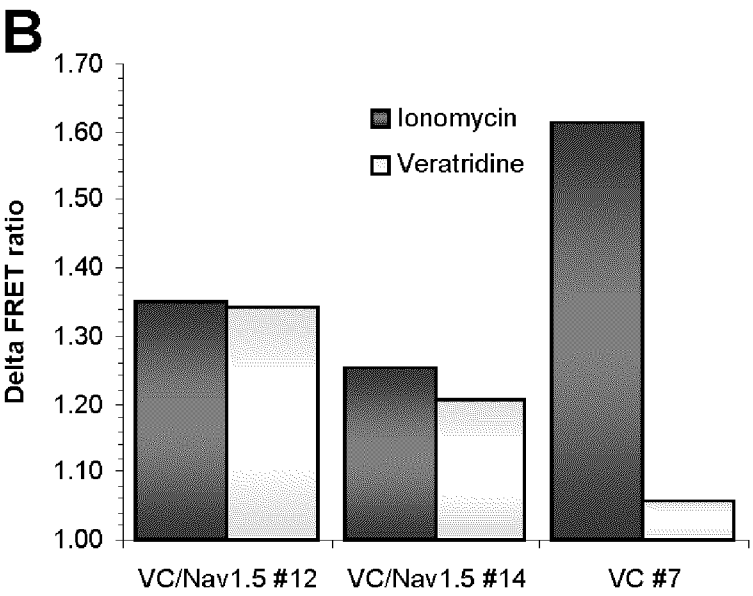


Fig. 4B

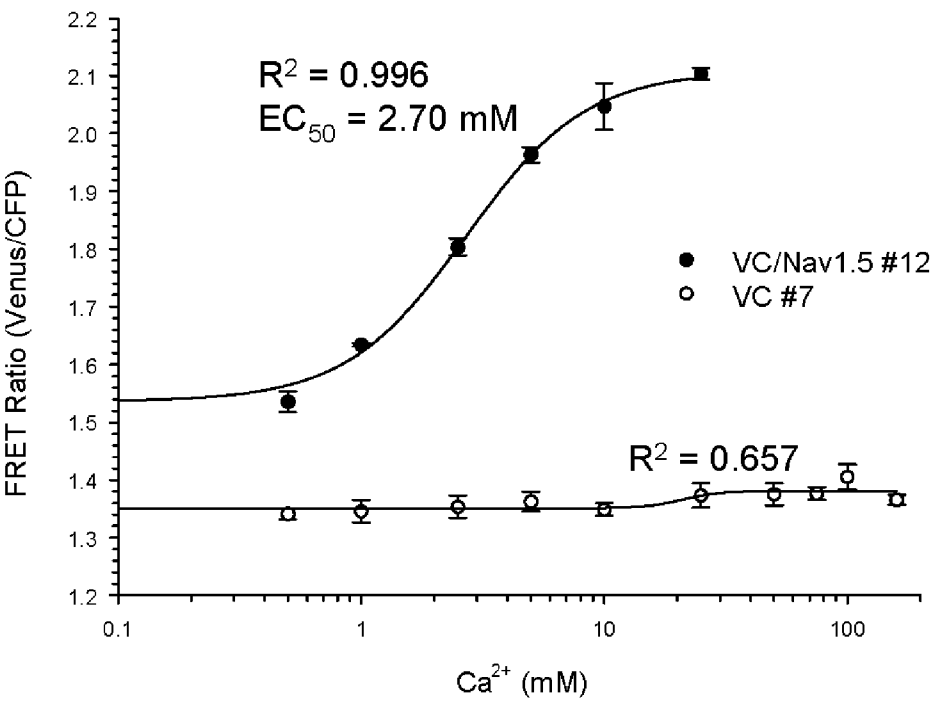


Fig. 5

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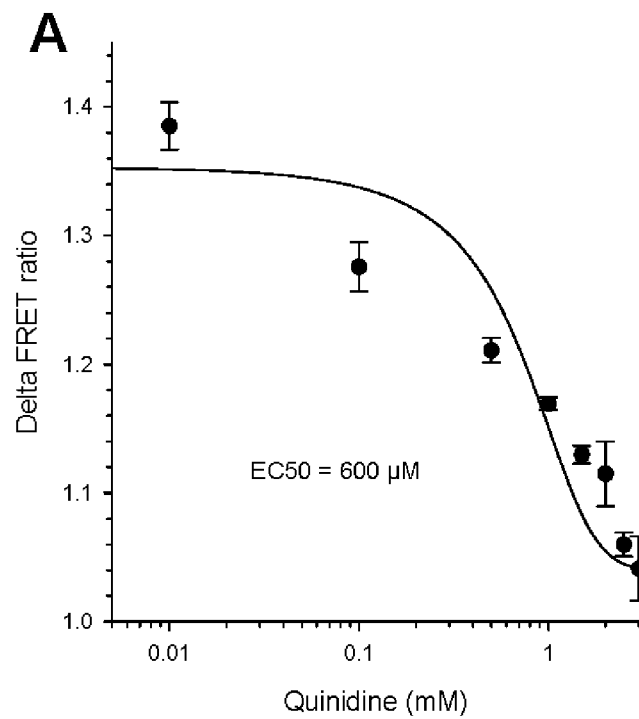


Fig. 6A

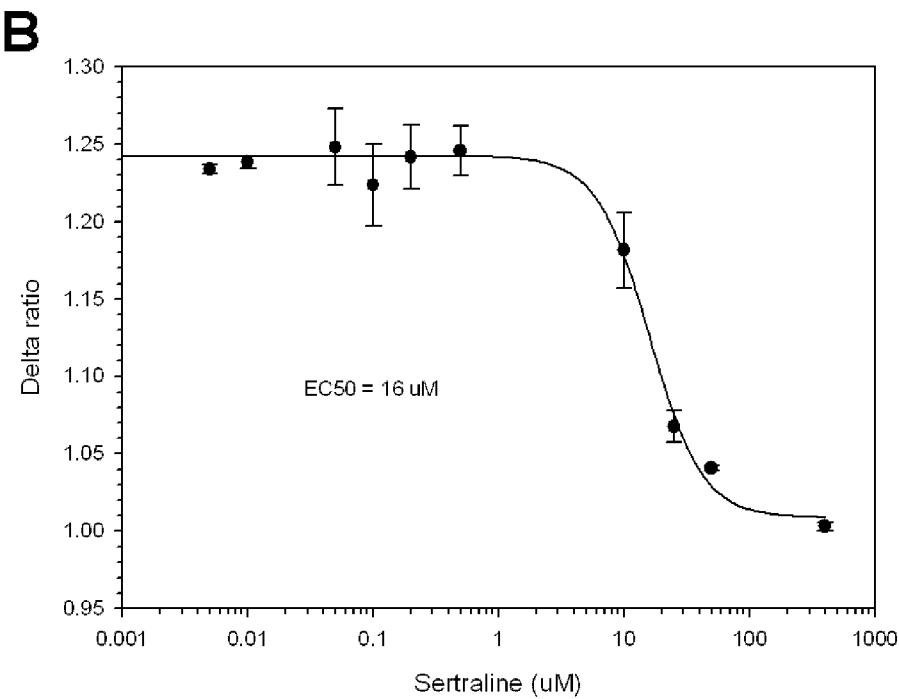


Fig. 6B

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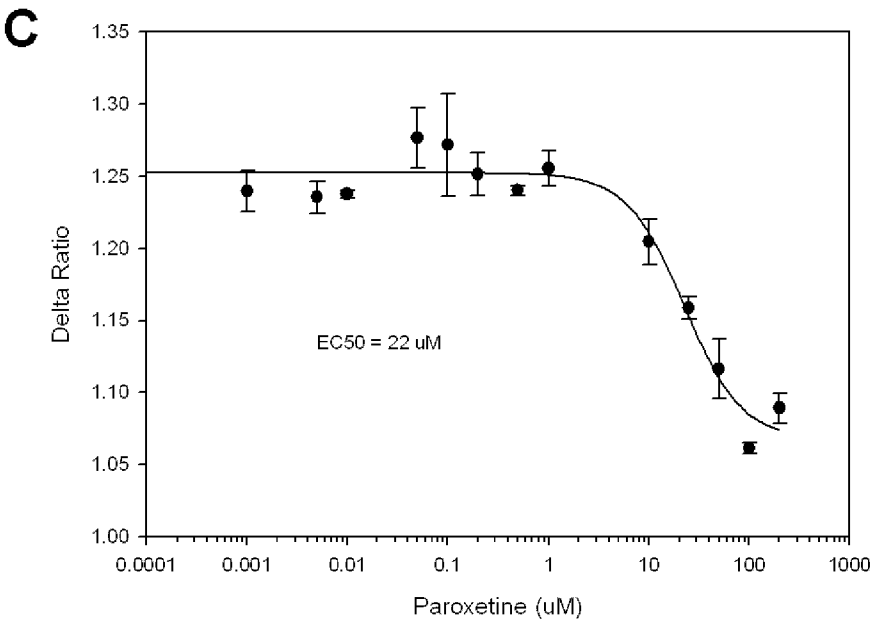


Fig. 6C

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Human Nav1.5^{mut}

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1  manfllprgt  ssfrrftres  laaiekrmae  kgargsttlq  esreglpeee  aprpqldlqa
61  skklpdlygn  ppqeligepl  edldpfystq  ktfivlnkgk  tifrfsatna  lyvlspfhpi
121 rraavkilvh  slfnmlimct  iltncvfmaq  hdpppwtkyv  eytftaiytf  eslvkilarg
181 fclhaftflr  dpwnwldfsv  iimayttefv  dlgnvsalrt  frvlralkti  svisgkktiv
241 galigsvkkl  advmvlvfc  lsvfaliglq  lfmgnlrhkc  vrnftalngt  ngsveadglv
301 wesldlylsd  penyllkngt  sdvllcgnss  dagtcpegyr  clkagenpdh  gytsfdfsaw
361 aflalfrlmt  qdowerlyqg  tlrsgakiym  iffmvlviflg  sfylvnlila  vvamayeegn
421 qatiaeteek  ekrfqeamem  lkkehealti  rgvdtvsrss  lemsplapvn  sherrskrrk
481 rmssgteecg  edrlpkdse  dgpramnhls  ltrglstsm  kprsrsgsif  tfrrrdlgse
541 adfaddenst  ageseshhts  llvpwplrrt  sagggpspgt  sapghalhgk  knstvdcnv
601 vsllgagdp  atspgshllr  pvmlehppdt  ttpseepggp  qmltsqapcv  dgfeepgarq
661 ralsavsvlt  saleeleesr  hkcpccwnrl  aqryliwec  plwmsikggv  klvmdpftd
721 ltitmciylv  tlfmalehyn  mtsefeemlq  vgnlvftgif  taemtfkiaa  ldpyyyfagg
781 wnifdsiivi  lslmelglsr  msnlsvlrsf  rllrvfklak  swptlntlik  iignsvgalg
841 ntlvlaiiv  fifavvgmql  fgknyseird  sdsgllprwh  mmdffhafli  ifrilcgewi
901 etmwdcmevs  gqslcllvfl  lvmvignlv  lnflallls  sfsadnltp  ddremnnlg
961 lalariqrgl  rfvrkrtwdf  ccgllrqrpq  kpaalaaagg  lpsciatpys  ppppetekvp
1021 ptrketrfee  geqpgggtpg  dpepvcvpia  vaesdtddqe  edeenslgte  eesskqgesq
1081 pvsggpeapp  dsrtwsqvsa  tasseaeasa  sqadwrqqwk  aepqapgcge  tpedscsegs
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1261 ygfkkyftna  wcwldflivd  vslyslvant  lgfaempik  slrtlrarp  lralsrfegm
1321 rvvnalvga  ipsimnvlv  clifwlifsi  mgvnlfagkf  grcinqtegd  lplnytivnn
1381 ksqceslnlt  gelywtkvkv  nfdnvgagyl  allqvafg  wmdimyaavd  srgyeeqpw
1441 eynlymyiyf  vifiifgsff  tlnlfigvii  dnfnqqkkkl  ggqdiqmtee  qkkyynamkk
1501 lsgkpkqkpi  prplnkyqgf  ifdivtkqaf  dvtimflicl  nmvtmmvetd  dgspekinil
1561 akinllfvai  ftgecivkla  alrhyyftns  wnifdfvvi  lsivgtvlsd  iiqkyffspt
1621 lfrvirlari  grilrlirga  kgirtllfal  mmslpalfni  gllflvmfi  ysifgmanfa
1681 yvkweagidd  mfnfgtfans  mlclfgitts  agwdgllspi  lntgppycdp  tlpnsngsrg
1741 dcgspavgil  fftyiisf  livvmyiai  ilenfsvate  esteplsedd  fdmfyeiwek
1801 fdpeatqfie  ysvlsdfada  lseplriakp  nqislinmdl  pmvsgdrihc  mdilfaftkr
1861 vlgesgemda  lkiqmeekfm  aanpskisye  pitttlrrkh  eevsamviqr  afrhllqrs
1921 lkhafllfrq  qagsglseed  aperegliay  vmsenfsrpl  gppssssiss  tsfppsdsdv
1981 tratsdnlqv  rgsdyshsed  ladfppspdr  dresiv

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Fig. 7A

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Human Nav1.5 alpha, isoform a (GenBank accession No. NP_932173)

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1  manfllprgt  ssfrrftres  laaiekmae  kqargsttlq  esreglpeee  aprpqldlqa
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121 rraavkilvh  slfnmlimct  iltncvfmag  hdpppwtkyv  eytftaiytf  eslvkilarg
181 fclhaftflr  dpwnwldfsv  iimayttefv  dlgnvsalrt  frvlralkti  svisglktiv
241 galiqsvkkl  advmvlvfc  lsvfaliglq  lfmgnlrhkc  vrnftalngt  ngsveadglv
301 wesldlylsd  penyllkngt  sdvllcgnss  dagtcpegys  clkagenpdh  gytsfdfsaw
361 aflalfrlmt  qdwerlygq  tlrsagkiym  iffmlviflg  sfylvnlila  vvamayeeqn
421 qatiaeteek  ekrfgeamem  lkkehealti  rgvdtvsrss  lemsplapvn  sherrskrrk
481 rmsgteecg  edrlpkdsd  dgpramnhls  ltrglstrsm  kprssrgsif  tfrrrdlgse
541 adfaddenst  ageseshhts  llvpwplrrt  saaggqpsgt  sapghalhgc  knstvdcnv
601 vsllgagdp  atspgshllr  pvmlehppdt  ttpseepggp  qmltsqapcv  dgfeepgarq
661 ralsavsvlt  saleeleesr  hkcpcwnrl  aqryliwecc  plwmsikqgv  klvmdpftd
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1021 ptrketrfee  geqpgggtpg  dpepvcvpia  vaesdtddge  edeenslgte  eesskqgesq
1081 pvsggpeapp  dsrtwsqvsa  tasseaeasa  sqadwrqgwk  aepqapgcge  tpedscsegs
1141 tadmtntael  leqipdlgqd  vkdpedcfte  gcvrpcpcca  vdttgapgkv  wwrlrktych
1201 ivehswfetf  iifmillssg  alafediyle  erktikvll  yadkmftyvf  vlemllkwva
1261 ygfkkyftna  wcwldflivd  vslvslvant  lgfaemgpik  slrtlrarp  lralsrfegm
1321 rvvnalvga  ipsimvllv  clifwlifsi  mgvnlfagkf  grcinqtegd  lplnytivnn
1381 ksqceslnlt  gelywtkvkv  nfdnvagayl  allqvafkq  wmdimyaavd  srgyeeqpgw
1441 eynlymyiyf  vifiifgsff  tlnlfigvii  dnfnqgkkkl  ggqdifmtee  qkkyynamkk
1501 lgskkpqkpi  prplnkyqgf  ifdivtkqaf  dvtimflicl  nmvtmmvetd  dqspekinil
1561 akinllfvai  ftgecivkla  alrhyyftns  wnifdfvvvi  lsivgtvlsd  liqkyffspt
1621 lfrvirlari  grilrlirga  kgirtllfal  mmslpalfni  glillflvmfi  ysifgmanfa
1681 yvkweagidd  mfnfqtfans  mlclfqitts  agwdgllspi  lntgppycdp  tlpnsngsrg
1741 dcgspavgil  fftyiiisf  livvnmiai  ilenfsvate  esteplsedd  fdmfyeiwek
1801 fdpeatqfie  ysvlsdfada  lseplriakp  nqislinmdl  pmvsgdrihc  mdilfaftkr
1861 vlgesgemda  lkiqmeekfm  aanpskisye  pitttlrrkh  eevsamviqr  afrhllqrs
1921 lkhafllfrq  qagsglseed  aperegliay  vmsenfsrpl  gppssssiss  tsfppsyzsv
1981 tratsdnlqv  rgsdyshsed  ladfppspdr  dresiv

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Fig. 7B

11229.297

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Human Na_v1.5 alpha, isoform b (GenBank accession No. NP_000326)

```

1  manfllprgt  ssfrrftres  laaiekmae  kqargsttlq  esreglpjee  aprpqldlqa
61  skklpdygn  ppqeligep  edldpfystq  ktfivlnkgk  tifrfsatna  lyvlspfhpi
121 rraavkilvh  slfnmlimct  iltncvfmaq  hdpppwtkyv  eytftaiytf  eslvkilarg
181 fclhaftflr  dpwnwldfsv  iimayttefv  dlgnvsalrt  frvlralkti  svisgkltiv
241 galiqsvkkl  advmvltvfc  lsvfaliglq  lfmgnlrhkc  vrnftalngt  ngsveadglv
301 wesldlylsd  penyllkngt  sdvllcgnss  dagtcepegr  clkagenpdh  gytsfdfsaw
361 aflalfrlmt  qdcwerlyqg  tlrsagkiym  iffmlviflg  sfylvnllla  vvamayeeqn
421 qatiaeteek  ekrfqeame  lkkehealti  rgvdtvsrss  lemsplapvn  sherrskrrk
481 rmssgteecg  edrlpkdsde  dgpramnhls  ltrglrstm  kprssrgsif  tfrrrdlgse
541 adfaddenst  ageseshhts  llvpwplrrt  saaggqspgt  sapghalhgc  knstvdcnqv
601 vsllgagdp  atspgshllr  pvmlehpdt  ttpseepggp  qmltsqapcv  dgfeepgarq
661 ralsavsvlt  saleeelesr  hkcpcwnrl  agryliwec  plwmsikqgv  klvmdpftd
721 ltitmcivln  tlfmalehyn  mtsefeemlg  vgnlvftgif  taentfkiia  ldpyyyfqqg
781 wnifdsiivi  lslmelglr  msnlsvlrsf  rllrvfklak  swptlntlik  iignsvgalg
841 nltlvaliiv  fifavvgmql  fgknyseird  sdsgllprwh  mmdffhaffli  ifrilcgewi
901 etmwdcmevs  gqslcllvfl  lvmvignlv  lnflalalls  sfsadnlta  dedremnlq
961 lalariqrgl  rfvrkrtwdf  ccgllrqrpq  kpaalaaggq  lpsciatpys  ppppetekvp
1021 ptrketrf  ee  geqpgqgt  pg  dpepvcv  pia  vaesdtdd  qe  edeenslg  te  eesskqes  qp
1081 vsggpeapp  d  srtwsqvs  at  asseaeas  as  qadwrqgw  ka  epqapgc  get  pedscseg  st
1141 admtntaell  eqipdlgg  dv  kdpedcft  eg  cvrrcpcc  av  dttgapgk  vw  wrlrktcy  hi
1201 vehswfetfi  ifmillss  ga  lafediyle  ee  rktikvlle  y  adkmftyv  fv  lemllkwv  ay
1261 gfkkyftnaw  cwldfliv  dv  slvslvant  l  gfaemgpik  s  lrtlrall  rl  ralsrfeg  mr
1321 vvnalvgai  psimnvll  vc  lifwlifsim  g  vnlfagkf  g  rcinqteg  dl  plnytivnn  k
1381 sqceslnltg  elywtkvk  vn  fdnvagay  la  llqvattf  kgw  mdimyaav  ds  rgyeeqpq  we
1441 ynlymyiyfv  ifiifgsf  ft  lnlfigvi  id  nfnqgkkk  lg  gqdiifm  tee  kkyynamk  kl
1501 gskkpqkpi  p  rplnkygg  fi  fdivtkqaf  d  vtimfli  cln  mvtmmvet  dd  qspekinil  a
1561 kinllfvaif  tgecivkl  aa  lrhyyftns  w  nifdfvvv  il  sivgtvls  di  iqkyffspt  l
1621 frvirlarig  rilrlirg  ak  girtllfal  m  mslpalfn  ig  lllflvmfi  y  sifgmanfa  y
1681 vkweagiddm  fnfgtfan  sm  lclfqitts  a  gwdgllsp  il  ntgppycd  pt  lpnsngsrg  d
1741 cgspavgilf  ftyiiisf  l  ivnmyiai  i  lenfsvate  ee  steplsedd  f  dmfyeiwe  kf
1801 dpeatqfley  svlsdfad  al  seplriak  pn  qislinmd  lp  mvsgdrih  cm  dilfaftkr  v
1861 lgesgemdal  kiqmeekf  ma  anpskisy  ep  itttlrrk  he  evsamviqu  ra  frrhllqrs  l
1921 khasflfrqg  agsglse  da  peregliay  v  msenfsrc  plg  ppsssssis  st  sfppsydsv  t
1981 ratsdnlqvr  gsdyshsed  l  adfppspdr  d  resiv

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Fig. 7C

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Human Nav1.5 alpha, isoform c (GenBank accession No. NP_001092874)

```

1  manfllprgt  ssfrrftres  laaiekmae  kgargsttlq  esreglpeee  aprpqldlqa
61  skklpdlygn  ppqeligapl  edldpfystq  ktfivlnkgk  tifrfsatna  lyvlspfhpi
121 rraavkilvh  slfnmlimct  iltncvfmaq  hdpppwtkyv  eytftaiytf  eslvkilarg
181 fclhaftflr  dpwnwldfsv  iimayvseni  klgnlsalrt  frvlralkti  svipglktiv
241 galiqsvkkl  advmvltvfc  lsvfaliglq  lfmgnlrhkc  vrnftalngt  ngsveadglv
301 wesldlylsd  penyllkngt  sdvllcgnss  dagtcpegys  clkagenpdh  gytsfdfsaw
361 aflalfrlmt  qdwerlyqg  tlrsagkiym  iffmlviflg  sfylvnllla  vvamayeeqn
421 qatiaeteek  ekrfgeamem  lkkehealti  rgvdtvsrss  lemsplapvn  sherrskrrk
481 rmssgteecg  edrlpkdsde  dgpramnhls  ltrglstrsm  kprssrgsif  tfrrrdlgse
541 adfaddenst  ageseshhts  llvpwplrrt  saqgqpspgt  sapghalhgk  knstvdcnv
601 vsllgagdp  atspgshllr  pvmlehppdt  ttpseepggp  qmltsqapcv  dgfeepgarq
661 ralsavsvlt  saleeleesr  hkcpcwnrl  aqryliwecc  plwmsikqgv  klvmdpftd
721 ltitmciylv  tlfmalehyn  mtsefeemlq  vgnlvftgif  taentfkiaa  ldpyyyfqqg
781 wnifdsiivi  lslmelglsl  msnlsvlrsf  rllrvfklak  swptlntlik  iignsvgalg
841 nltlvlaiiv  fifavvgmql  fgknyseird  sdsgllprwh  mmdffhaffli  ifrilcgewi
901 etmwdcnvsv  gqslcllvfl  lvmvignlvv  lnflalalls  sfsadnltp  dedremnnlq
961 lalariqrql  rfvrkrttwdf  ccgllrqrpq  kpaalaaqgq  lpsciatpys  ppppetekvp
1021 ptrketrfee  geqpgggtpg  dpepvcvpia  vaesdtddge  edeenslgte  eesskqgesq
1081 pvsggpeapp  dsrtwsqvsa  tasseaeasa  sqadwrqgwk  aepqapgcge  tpedscsegs
1141 tadmtntael  leqipdlgqd  vkdpedcfte  gcvrrcpcca  vdttgapgkv  wwrlrkctyh
1201 ivehswfetf  iifmillssg  alafediyle  erktikvll  yadkmftyvf  vlemllkwva
1261 ygfkkyftna  wcwldflivd  vslvslvant  lgfaemgpik  slrtlrarp  lralsrfegm
1321 rvvnalvga  ipsimvllv  clifwlifsi  mgvnlfagkf  grcinqtegd  lplnytivnn
1381 ksqceslnlt  gelywtkvkv  nfdnvgagyl  allqvafkq  wmdimyaavd  srgyeegpqw
1441 eynlymyiyf  vifiifgsff  tlnlfigvii  dnfnqgkkkl  ggqdifmtee  qkkyynamkk
1501 lgskkpqkpi  prplnkyqgf  ifdivtkqaf  dvtimflicl  nmvtmmvetd  dqspekinil
1561 akinllfvai  ftgecivkla  alrhyyftns  wnifdfvvvi  lsivgtvlsl  liqkyffspt
1621 lfrvirlari  grilrlirga  kgirtllfal  mmslpalfni  gllflvmfi  ysifgmanfa
1681 yvkweagidd  mfnfqtfans  mlclfqitts  agwdgllspi  lntgppycdp  tlpnsngsrg
1741 dcgspavgil  fftyiiiisf  livvnmiai  ilenfsvate  esteplsedd  fdmfyeiwek
1801 fdpeatqfie  ysvlsdfada  lseplriakp  ngislinmdl  pmvsgdrihc  mdilfaftkr
1861 vlgesgemda  lkiqmeekfm  aanpskisye  pitttlrrkh  eevsamviqr  afrhllqrs
1921 lkhafllfrq  qagsglseed  aperegliay  vmsenfsrpl  gppssssiss  tsfppsdydv
1981 tratsdnlqv  rgsdyshsed  ladfppspdr  dresiv

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Fig. 7D

11229.297

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Human Nav1.5 alpha, isoform d (GenBank accession No. NP_001092875)

```

1  manfllprgt  ssfrrftres  laaiekmae  kqargsttlq  esreglpeee  aprpqldlga
61  skklpdlygn  ppqeligepl  edldpfystq  ktflvlnkgk  tifrfsatna  lyvlspfhpi
121 rraavkilvh  slfnmlimct  iltncvfmag  hdpppwtkyv  eytftaiytf  eslvkilarg
181 fclhaftflr  dpwnwldfsv  iimayvseni  klgnlsalrt  frvlralkti  svipglktiv
241 galigsvkkl  advmvltvfc  lsvfaliglq  lfmgnlrhkc  vrnftalngt  ngsveadglv
301 wesldlylsd  penyllkngt  sdvllcgns  dagtcegyr  clkagenpdh  gytsfdfsaw
361 aflalfrlmt  qdcwerlyqg  tlrsagkiym  iffmlviflg  sfylvnlila  vvamayeeqn
421 qatiaeteek  ekrfqeamem  lkkehealti  rgvdtvsrss  lemsplapvn  sherrskrrk
481 rmssgteecg  edrlpkdsde  dgpramnhls  ltrglstrsm  kprssrgsif  tfrrrdlgse
541 adfaddenst  ageseshhts  llvpwplrrt  saqggqspgt  sapghalhgk  knstvdcong
601 vsllgagdpe  atspgshllr  pvmlehppdt  ttpseepggp  qmltsqapcv  dgfeepgarq
661 ralsavsvlt  saleeleesr  hkcppcwnrl  aqryliwec  plwmsikqgv  klvmdpftd
721 ltitmciylv  tlfmalehyn  mtsefeemlg  vgnlvftgif  taemtfskia  ldpyyyyfqg
781 wnifdsiivi  lsmlgelgr  msnlsvlrsf  rllrvfklak  swptlntlik  iignsvgalg
841 nltlvoliiv  fifavvgmql  fgknyseird  sdsgllprwh  mmdffhafli  ifrilcgewi
901 etmwdcmevs  ggsllcllvfl  lvmvignlv  lnflalalls  sfsadnltp  dedremnnlq
961 lalariqrql  rfvrkrttdf  cggllrqrpq  kpaalaaagg  lpsciatpys  ppppetekvp
1021 ptrketrf  ee  geqpgggtpg  dpepvcvpia  vaesdtddqe  edeenslgte  eesskqgesq
1081 pvsggpeapp  dsrtwsqvsa  tasseaeasa  sqadwrqgwk  aepqapgcge  tpedscsegs
1141 tadmtntael  legipdlgqd  vkdpedcfte  gcvrpcpcca  vdttgapgkv  wwrlrktcyh
1201 ivehswfetf  iifmillssg  alafediyle  erktikvll  yadkmftyvf  vlemllkwva
1261 ygfkkyftna  wcwldflivd  vslvslvant  lgfaemgpik  slrtlralrp  lralsrfegm
1321 rvvnalvga  ipsimnvllv  clifwlifsi  mgvnlfagkf  grcinqtegd  lplnytivnn
1381 ksqceslnlt  gelywtkvkv  nfdnvgagyl  allqvyeegp  qweynlymyi  yfvifiifgs
1441 fftlnlfigv  iidnfnqqkk  klggqdifmt  eeqkkyynam  kklgskkpqk  piprplnkyq
1501 gfifdivtkq  afdvtimfli  clnmvmtmve  tddqspekin  ilakinllfv  aiftgecivk
1561 laalrhyyft  nswnifdfv  vilsivgtvl  sdiigkyffs  ptlfrvirla  rigrilrlir
1621 gakgirtllf  almmslpalf  niglllflvm  fiysifgman  fayvkweagi  ddmfnfqtfa
1681 nsmlclfqit  tsagwdglls  pilntgppyc  dptlpnsngs  rgdcgspav  ilfftttyiii
1741 sfliivnmyi  aiilenfsva  teesteplse  ddfdmfyew  ekfdpeatqf  ieysvlsdfa
1801 dalseplria  kpnqislinm  dlpmvsgdri  hcndilfaft  krvlgesgem  dalkiqmeek
1861 fmaanpskis  yepitttlrr  kheevsamvi  grafrhrllq  rslkhasflf  rqqagsglse
1921 edaperegli  ayvmsefnsr  plgppssssi  sstsfppsyd  svtratsdnl  qvrgsdyshs
1981 edladfpssp  drdresiv

```

Fig. 7E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001838

A. CLASSIFICATION OF SUBJECT MATTER IPC: C07K 14/705 (2006.01) , C07K 14/47 (2006.01) , C12N 15/12 (2006.01) , C12Q 1/02 (2006.01) , G01N 33/567 (2006.01) , G01N 21/64 (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols) (2006.01) C07K 14/705, C07K 14/47, C12N 15/12, C12Q 1/02, G01N 33/567, G01N 21/64																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																						
Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Canadian Patent Database (CPD), SCOPUS, Delphion, Genomequest, Pubmed, Google key words: Nav1.5 alpha, SCN5A, hH1, LQT3, Brugada syndrome, sodium channel, mutation, chimeric, calmodulin, myosin, calcium,																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
Y	BAROUDI et al. Expression and intracellular localization of an <i>SCN5A</i> double mutant R1232W/T1620M implicated in Brugada syndrome. CIRCULATION RESEARCH. 2002. Vol. 90, pages e11-e15. ISSN 0009-7330. *whole document*	1-39																				
Y	FAVRE et al. On the structural basis for ionic selectivity among Na ⁺ , K ⁺ , and Ca ²⁺ in the voltage-gated sodium channel. BIOPHYSICAL JOURNAL. 1996. Vol. 71, pages 3110-3125. ISSN: 0006-3495. *whole document* *page 3112, column 2; last paragraph* *pages 3115-3120* *tables 2 and 3*	1-39																				
[X] Further documents are listed in the continuation of Box C. [] See patent family annex.																						
* Special categories of cited documents : <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
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"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 08 February 2010 (08-02-2010)		Date of mailing of the international search report 23 March 2010 (23-03-2010)																				
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Claire Plumb																				

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2009/001838**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. (means)
☐ on paper
☒ in electronic form
 - b. (time)
☐ in the international application as filed
☒ together with the international application in electronic form
☐ subsequently to this Authority for the purposes of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments :

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001838

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEST et al. A cluster of hydrophobic amino acid residues required for fast Na ⁺ -channel inactivation. PROC NATL ACAD SCI U S A. 1992. Vol. 89, pages 10910-10914. ISSN: 0027-8424. *cited by applicant* *whole document*	1-39
Y	MIYAKAWAKI et al. Fluorescent indicators for Ca ²⁺ based on green fluorescent proteins and calmodulin. NATURE. 1997. Vol. 388, pages :882-887. ISSN: 0028-0836. *cited by applicant* *whole document*	18-39
Y	IKURA et al. Solution structure of a calmodulin-target peptide complex by multidimensional NMR. SCIENCE. 1992. Vol. 256, pages 632-638. ISSN: 0193-4511. *cited by applicant* *abstract* *page 633, first column, last paragraph*	18-39
Y, P	BISWAS et al. Calcium-mediated dual-mode regulation of cardiac sodium channel gating. CIRCULATION RESEARCH. 2009. Vol. 104, pages 870-878. ISSN 0009-7330. *whole document*	1-39