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(54) Title: METHODS AND COMPOSITIONS FOR MEASURING ION CHANNEL CONDUCTANCE

(57) Abstract: The invention relates to novel methods for measuring ion channel transmission and methods and compositions useful in the identification of ligand gated channel agonists and modulators.

Methods and Compositions for Measuring Ion Channel Conductance

FIELD OF THE INVENTION

The invention relates to a novel methods for measuring cellular ion channel transmission and methods and compositions useful in the identification of ligand gated ion channel agonists and modulators.

BACKGROUND OF THE INVENTION

Ion Channels

Ion channel proteins form hydrophilic pores that extend across the cellular lipid bilayer; when these pores are open, they allow specific molecules (usually inorganic ions of appropriate size and charge) to pass through them and thereby cross the membrane.

Channel proteins which are concerned specifically with inorganic ion transport are referred to as ion channels, and include ion channels for sodium, potassium, calcium, and chloride ions. Ion channels which open in response to a change in the voltage across the membrane are referred to as voltage gated ion channels (or voltage-dependent ion channels). Ion channels which open in response to the binding of a ligand to the channel protein are referred to as ligand gated ion channels.

The present invention describes new ion channels and provides methods and compositions suitable for high throughput screening of ion channels.

DESCRIPTION OF THE INVENTION

Voltage Gated Ion Channels

Voltage Gated Sodium Channel

Voltage gated ion channels are a class of channel proteins that play a major role in cellular electrical excitability. In the majority of excitable tissues, the early depolarization phase of action potentials is mediated by a sodium current via voltage-dependent sodium channels (also known as voltage-gated sodium channels or VGSCs). The sodium channel is one of the most thoroughly characterized of the voltage gated channels.

The primary structures of many sodium channels from a variety of tissues (brain, skeletal muscle and cardiac muscle) and organisms (jellyfish, squid, eel, rat, human) have been identified, and their amino acid sequences show individual regions which are highly conserved over evolution, indicating that

voltage-dependent sodium channels belong to a large superfamily of evolutionarily related proteins. All published polypeptide complexes of VGSCs have in common a large, about 260 kDa glycoprotein (the pore forming subunit) which is called the alpha subunit (Agnew et al. 1978; Agnew et al. 1980; Catterali 1986; Catterall 1992). Additional lower molecular weight polypeptides, the beta-subunits, have been found to be associated with sodium channels from mammalian muscle (Kraner et al. 1985; Tanaka et al. 1983) and brain (Hartshorne and Catterall 1984). The large, pore-forming alpha subunit is sufficient for all known functions of VGSCs (Catterall 1992) while the beta subunits modulate some of the functions of the alpha subunit (Catterall 1992).

Voltage Gated Potassium Channels

Voltage-gated potassium channels make up a large molecular family of integral membrane proteins that are fundamentally involved in the generation of bioelectric signals such as nerve impulses. These proteins span the cell membrane, forming potassium-selective pores that are rapidly switched open or closed by changes in membrane voltage. Several chemical entities have been discovered to be potent and specific openers of vascular potassium K⁺ channels. These include cromakalim and its derivatives and RP 52891. This mechanism is also shared, at least partially, by drugs such as minoxidil, diazoxide, pinacidil and nicorandil. The opening of plasmalemmal K⁺ channels produces loss of cytosolic K⁺. This effect results in cellular hyperpolarization and functional vasorelaxation. In normotensive or hypertensive rats, K⁺ channel activators decrease aortic blood pressure (by producing a directly mediated fall in systemic vascular resistance) and reflexively increase heart rate. K⁺ channel openers produce selective coronary vasodilatation and afford functional and biochemical protection to the ischemic myocardium.

The structure of a typical voltage-gated potassium channel protein is known to be comprised of six membrane spanning domains in each subunit, each of which is regulated by changes in membrane potential. B. Hille. "Ionic Channels of Excitable Membranes"(Sinauer, Sunderland, Mass., 1992). Voltage-gated potassium channels sense changes in membrane potential and move potassium ions in response to this alteration in the cell membrane potential. Molecular cloning studies on potassium channel proteins has yielded information primarily for members of the

voltage-gated family of potassium channels. Various genes encoding these voltage-gated family of potassium channel proteins have been cloned using *Drosophila* genes derived from both the Shaker, Shaw and Shab loci; Wei, A. et. al., Science (1990) Vol. 248 pp. 599-603.

5 **Voltage Gated Calcium Channels**

Voltage-gated calcium channels are present in neurons, and in cardiac, smooth, and skeletal muscle and other excitable cells. These channels are known to be involved in membrane excitability, muscle contraction, and cellular secretion, such as in exocytotic synaptic transmission (McCleskey, et al., 1987). In neuronal cells, voltage-gated calcium channels have been classified by their electrophysiological as well as by their biochemical (binding) properties.

Calcium channels are generally classified according to their electrophysiological properties as Low-voltage-activated (LVA) or High-voltage-activated (HVA) channels. HVA channels are currently known to comprise at least three groups of channels, known as L-, N- and P-type channels (Nowycky, et al., 1985). These channels have been distinguished one from another structurally and electrophysiologically as well as biochemically on the basis of their pharmacology and ligand binding properties. Thus, dihydropyridines, diphenylalkylamines and piperidines bind to the α_1 subunit of the L-type calcium channel and block a proportion of HVA calcium currents in neuronal tissue, which are termed L-type calcium currents.

N- or omega-type HVA calcium channels are distinguishable from other calcium channels by their sensitivity to omega conotoxins (omega conopeptides). Such channels are insensitive to dihydropyridine compounds, such as L-type calcium channel blockers nimodipine and nifedipine. (Sher and Clementi, 1991).

Ligand Gated Ion Channel Receptors

Ligand-gated ion channels provide a means for communication between cells of the central nervous system. These channels convert a signal (e.g., a chemical referred to as a neurotransmitter) that is released by one cell into an electrical signal that propagates along a target cell membrane. A variety of neurotransmitters and neurotransmitter receptors exist in the central and peripheral nervous systems. At the present time, numerous families of ligand-gated receptors have been identified and characterized on the basis of sequence

identity these include nicotinic acetylcholine, glutamate, glycine, GABA A, 5-HT₃, and the purinoceptors. These can be further characterized by whether the gated ion channel transmits cations or anions. Those which form cationic channels include, for example, excitatory nicotinic acetylcholine receptors (nAChRs),
5 excitatory glutamate-activated receptors, the 5-HT₃ serotonin receptor, and the purine receptor.

Those which form anionic channels include, for example, the inhibitory GABA and glycine-activated receptors. This discussion will confine itself to those ligand gated ion channel receptors which conduct cations.

10 **5HT₃ Receptor**

Molecular cloning has indicated that serotonin (5-hydroxytryptamine, also referred to as 5-HT) receptors belong to at least two protein superfamilies:

G-protein-associated receptors and ligand-gated ion channel. The 5-HT₃ receptor belongs to the family of ligand-gated ion channels. As discussed below the 5-HT₃
15 receptor is primarily a sodium potassium ligand gated ion channel under physiologic conditions. The inflammatory and painproducing effects of serotonin are generally believed to be mediated via 5HT₃ receptors on peripheral sensory endings (Richardson, B. P., et al., 1985).

Nicotinic Receptors

20 The nicotinic acetylcholine receptors (nAChRs) are multisubunit proteins of neuromuscular and neuronal origins. These receptors form ligand-gated ion channels that mediate synaptic transmission between nerve and muscle and between neurons upon interaction with the neurotransmitter acetylcholine (ACh). Since various nicotinic acetylcholine receptor (nAChR) subunits exist, a variety
25 of nAChR compositions (i.e., combinations of subunits) exist. The different nAChR compositions exhibit different specificities for various ligands and are thereby pharmacologically distinguishable. Thus, the nicotinic acetylcholine receptors expressed at the vertebrate neuromuscular junction in vertebrate sympathetic ganglia and in the vertebrate central nervous system have been
30 distinguished on the basis of the effects of various ligands that bind to different nAChR compositions. For example, the elapid alpha -neurotoxins that block activation of nicotinic acetylcholine receptors at the neuromuscular junction do not block activation of some neuronal nicotinic acetylcholine receptors that are expressed on several different neuron-derived cell lines.

Muscle nAChR is a glycoprotein composed of five subunits with the stoichiometry $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$. Each of the subunits has a mass of about 50-60 kilodaltons (kd) and is encoded by a different gene. The $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$ complex forms functional receptors containing two ligand binding sites and a ligand-gated transmembrane channel. Upon interaction with a cholinergic agonist, muscle nicotinic AChRs conduct sodium ions. The influx of sodium ions rapidly short-circuits the normal ionic gradient maintained across the plasma membrane, thereby depolarizing the membrane. By reducing the potential difference across the membrane, a chemical signal is transduced into an electrical signal that signals muscle contraction at the neuromuscular junction.

Functional muscle nicotinic acetylcholine receptors have been formed with $\alpha\beta\delta\gamma$ subunits, $\alpha\beta\gamma$ subunits, $\alpha\beta\delta$ subunits, $\alpha\beta\gamma$ subunits or $\alpha\delta$ subunits, but not with only one subunit (see e.g., Kurosaki et al. 1987; Camacho et al. 1993). In contrast, functional neuronal AChRs (nAChRs) can be formed from α subunits alone or combinations of α and β subunits. The larger α subunit is generally believed to be the ACh-binding subunit and the lower molecular weight β subunit is generally believed to be the structural subunit, although it has not been definitively demonstrated that the β subunit does not have the ability to bind ACh. Each of the subunits which participate in the formation of a functional ion channel are, to the extent they contribute to the structure of the resulting channel, "structural" subunits, regardless of their ability (or inability) to bind ACh.

Neuronal AChRs (nAChRs), which are also ligand-gated ion channels, are expressed in ganglia of the autonomic nervous system and in the central nervous system (where they mediate signal transmission), in post-synaptic locations (where they modulate transmission), and in pre- and extra-synaptic locations (where they may have additional functions). The nAChRs comprise a large family of neurotransmitter regulated ion channels that control neuronal activity and brain function. These receptors have a pentameric structure. The gene family is composed of nine α and four β subunits that co-assemble to form multiple subtypes of receptors that have a distinctive pharmacology. Acetylcholine is the

endogenous regulator of all of the subtypes, while nicotine non-selectively activates all nAChRs. Known chemical templates have subtype selectivity.

$\alpha 7$ nAChR is a ligand-gated Ca^{++} channel formed by a homopentamer of $\alpha 7$ subunits. $\alpha 7$ nAChR is of particular interest because $\alpha 7$ nAChR agonists increase neurotransmitter release, increase cognition, arousal, attention, learning and memory. $\alpha 7$ nAChR is expressed at high levels in the hippocampus, ventral tegmental area and ascending cholinergic projections from nucleus basalis to thalamocortical areas. Previous studies have established that a α -bungarotoxin (α -btx) binds selectively to this homopentameric, $\alpha 7$ nAChR subtype, and that $\alpha 7$ nAChR has a high affinity binding site for both α -btx and methyllycaconitine (MLA). We have chosen to use $\alpha 7$ nAChR as a model system for high throughput drug screening

Glutamate Receptors

Glycine also functions in excitatory transmission by modulating the actions of glutamate, the major excitatory neurotransmitter in the central nervous system. (Johnson and Ascher, 1987)

Glutamate binds or interacts with one or more glutamate receptors which can be differentiated pharmacologically into several subtypes. In the mammalian central nervous system (CNS) there are three main subtypes of ionotropic glutamate receptors, defined pharmacologically by the selective agonists N-methyl-D-aspartate (NMDA), kainate (KA), and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). The NMDA receptor has been implicated in a variety of neurological pathologies including stroke, head trauma, spinal cord injury, epilepsy, anxiety, and neurodegenerative diseases such as Alzheimer's Disease (Watkins and Collingridge 1989). A role for NMDA receptors in nociception and analgesia has been postulated as well (Dickenson, 1990). More recently, AMPA receptors have been widely studied for their possible contributions to such neurological pathologies (Fisher and Bogousslavsky, 1993).

When activated by glutamate, the endogenous neurotransmitter, the NMDA receptor permits the influx of extracellular calcium (Ca^{++}) and sodium (Na^{+}) through an associated ion channel. The NMDA receptor allows considerably more influx of Ca^{++} than do kainate or AMPA receptors and is an example of a receptor-operated Ca^{++} channel. Normally, the channel is

opened only briefly, allowing a localized and transient increase in the concentration of intracellular Calcium (Ca^{++}) which, in turn, alters the functional activity of the cell.

The activity of the NMDA receptor-ionophore complex is regulated by a variety of modulatory sites that can be targeted by selective antagonists. Competitive antagonists, such as the phosphonate AP5, act at the glutamate binding site, whereas noncompetitive antagonists, such as phencyclidine (PCP), MK-801 or magnesium (Mg^{++}), act within the associated ion channel (ionophore). There is also a glycine binding site that can be blocked selectively with compounds such as 7-chlorokynurenic acid. There is evidence suggesting that glycine acts as a co-agonist, so that both glutamate and glycine are necessary to fully elicit NMDA receptor-mediated responses. Other potential sites for modulation of NMDA receptor function include a zinc (Zn^{2+}) binding site and a sigma ligand binding site. Additionally, endogenous polyamines such as spermine are believed to bind to a specific site and so potentiate NMDA receptor function (Ransom and Stec, 1988). The potentiating effect of polyamines on NMDA receptor function may be mediated via a specific receptor site for polyamines.

20 **Purinergic Receptors**

Purinergic receptors are classified as P1 (adenosine as ligand) and P2 (ATP as ligand). The P2 receptors are subclassified into two broad types-those that are 7-transmembrane receptors that couple to G-proteins (P_{2Y}, P_{2U}, P_{2T}, and perhaps P_{2Z}). Another major class of purinoceptors are the P_{2x} purinoceptors which are ligand-gated ion channels possessing intrinsic ion channels permeable to Na^+ , K^+ , and Ca^{++} . P_{2x} receptors described in sensory neurons are important for primary afferent neurotransmission and nociception. ATP is known to depolarize sensory neurons and plays a role in nociceptor activation since ATP released from damaged cells stimulates P_{2x} receptors leading to depolarization of nociceptive nerve-fiber terminals.

ATP-sensitive potassium channels have been discovered in numerous tissues, including kidney, vascular and non-vascular smooth muscle and brain, and binding studies using radiolabeled ligands have confirmed their existence. Opening of these channels causes potassium (K^+) efflux and hyperpolarizes the cell membrane

Ion Channels as Drug Targets

Ion channels both ligand gated and voltage gated, are in general excellent and validated drug targets. For some channels however, a functional high throughput screening assay is problematic because expression levels are low and function is hard to measure using standard detection technology for high throughput screening. For those channels which normally conduct a cation other than calcium high throughput screening methods are often cumbersome. For calcium conductance however, several rapid assays exist. It would often be desirable to This invention provides the scientist with a detailed description about how to convert a channel normally conducting sodium or potassium under physiologic conditions to one conducting calcium for ease in assay development.

The $\alpha 7$ nAChR discussed above is one ligand gated ion channel that has proved to be a difficult target for developing a functional high throughput screening assay. Native $\alpha 7$ nAChR are not routinely able to be stably expressed in most mammalian cell lines (Cooper and Millar 1997). Repeated attempts by our group to stably express the human $\alpha 7$ nAChR in HRK 293, CHO, COS and SH-EP1 were unsuccessful. While it was possible to identify cell lines that initially expressed functional $\alpha 7$ nAChR, these lines dramatically lost receptor expression with prolonged growth in culture. Under these conditions it was not possible to use these lines for screening purposes. Another feature that makes functional assays of $\alpha 7$ nAChR challenging is that the receptor is rapidly (100 milliseconds) inactivated agonist application. This rapid inactivation greatly limits the functional assays that can be used to measure channel activity

One solution to the problem is to engineer the $\alpha 7$ nAChR to have a longer duration of open probability and to have it be expressed better in mammalian cells. We are aware of a report indicating that a chimeric receptor formed between the N-terminal ligand binding domain of the $\alpha 7$ nAChR (AA 1-201) and the pore forming C-terminal domain of the 5-HT₃ receptor expressed well in *Xenopus* oocytes while retaining nicotinic agonist sensitivity (Eisele et al. 1993). Eisele et al (1993) used the N-terminus of the avian (chick) form of the $\alpha 7$ nAChR receptor and the c-terminus of the mouse form of the 5-HT₃ gene. The report of Eisele et. al. was interesting to us because we knew from our own studies that the 5-HT₃ channels expressed well in most mammalian cells. In addition, we also knew from past studies that 5-HT₃

channels inactivated much slower than nicotinic channels. A chimeric receptor prepared from the ligand binding region of $\alpha 7$ nAChR and the pore forming domain of 5-HT₃ might express well in mammalian cells and might be easier to measure in a functional assay. However, under physiological conditions the $\alpha 7$ nAChR is a calcium channel while the 5-HT₃ receptor is a sodium and potassium channel. Indeed, Eisele et al. teaches that the chicken $\alpha 7$ nAChR/ mouse 5-HT₃ receptor behaves quite differently than the native $\alpha 7$ nAChR with the pore element not conducting calcium but actually being blocked by calcium ions. The chicken/mouse hybrid of Eisele is also not suitable for accessing compounds for their activity at the human $\alpha 7$ nAChR receptor. The human $\alpha 7$ nAChR has 92% identity with the chicken $\alpha 7$ nAChR, but surprisingly, the pharmacology of the two receptors are different. For example, 1,1-dimethyl-4-phenylpiperazinium is a full agonist at the human receptor and a partial agonist at the chicken receptor (Peng et al 1994). Other large species-specific differences in binding affinity have been noted (Peng et al 1994).

Ligand binding can be accessed in either whole cells or membrane preparations but both kinds of assays are cumbersome. Whole cell assays have been difficult to perform in a high throughput screening format because of the extensive washing and manipulation required to obtain a good signal to noise ratio. Isolated membranes have been used in such assays but also typically require extensive manipulation to prepare the membranes themselves and the assay itself requires extensive manipulation and washing to obtain a favorable signal to noise ratio. Such assays are illustrated in US Patent No. 6,022,704. A binding assay which could be performed without such required extensive manipulation would be extremely useful.

Within the last few years very precise measurement of cellular fluorescence in a high throughput whole cell assay has become possible with the use of a device marketed by Molecular Devices, Inc. designated "FLIPR" (Schroeder et al. 1996), entire document, full reference provided below, incorporated herein by reference. FLIPR has shown considerable utility in measuring membrane potential of mammalian cells using voltage-sensitive fluorescent dyes but is useful for measuring essentially any cellular fluorescence phenomenon. The device uses low angle laser scanning illumination and a mask to selectively excite fluorescence within approximately 200 microns of the bottoms of the wells in standard 96 well plates. The low angle of the laser reduces background by selectively directing the light to the

cell monolayer. This avoids background fluorescence of the surrounding media. This system then uses a CCD camera to image the whole area of the plate bottom to measure the resulting fluorescence at the bottom of each well. The signal measured is averaged over the area of the well and thus measures the average response of a population of cells. The system has the advantage of measuring the fluorescence in each well simultaneously thus avoiding the imprecision of sequential measurement well by well measurement. The system is also designed to read the fluorescent signal from each well of a 96 or 384 well plate as fast as twice a second. This feature provides FLIPR with the capability of making very fast measurements in parallel. This property allows for the measurement of changes in many physiological properties of cells that can be used as surrogated markers to a set of functional assays for drug discovery. FLIPR is also designed to have state of the art sensitivity. This allows it to measure very small changes with great precision.

INFORMATION DISCLOSURE

US Patent No. 6,022,704, Feb. 8, 2000, DNA and mRNA encoding an alpha 4 subunit of human neuronal nicotinic acetylcholine receptor and cells transformed with same, Elliott, K. J. et. al.

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BRIEF DESCRIPTION OF THE FIGURES

- Figure 1. Construction of the $\alpha 7/5$ -HT₃ Chimeric Ligand Gated Ion Channel
- 10 Figure 2 Amino Acid Sequence of the mature cell surface form of the $\alpha 7/5$ -HT₃ Chimeric Ligand Gated Ion Channel. (mutant $\alpha 7$ receptors of SEQ ID NOS: 10, 12, 14) have same mature amino terminus)
Underlined = N-terminal AA (1-201) from human $\alpha 7$ nAChR gene
Not underlined = C-terminal AA from mouse 5-HT₃ gene
15 Bold font = position of transmembrane domain 1
- Figure 3 Fl-btx binding to the $\alpha 7/5$ -HT₃ Chimeric Ligand Gated Ion Channel
- Figure 4 Epibatidine Competes Fl-btx Binding to $\alpha 7/5$ -HT₃ Chimeric Ligand Gated Ion Channel
- 20 Figure 5 α -btx Competes Fl-btx Binding to $\alpha 7/5$ -HT₃ Chimeric Ligand Gated Ion Channel
- Figure 6 Non-Physiologic Buffer Increases Calcium Flux through the $\alpha 7/5$ -HT₃ Chimeric Ligand Gated Ion Channel
- 25 Figure 7 Non-Physiologic Buffer does not Increase the Bradykinin-Induced Calcium Flux
- 30 Figure 8 Exemplary Data from a screen for modulators of activity indicating a test compound is an antagonist
- Figure 9 Assay of function of double mutant human $\alpha 7$ ligand gated ion channel
- 35 Figure 10 Exemplary Data from a screen for modulators of activity indicating a test compound is an antagonist

Brief Description of the Sequence Listing

- 40 Sequence 1 DNA coding sequence of the wild type human $\alpha 7$ ligand gated ion channel
- Sequence 2 Amino acid sequence of the wild type human $\alpha 7$ ligand gated ion channel
- 45 Sequence 3 DNA coding sequence of the murine 5HT₃ ligand gated ion channel

- Sequence 4 Amino acid sequence of the murine 5HT₃ ligand gated ion channel
- 5 Sequence 5 DNA coding sequence of the human $\alpha 7$ /murine 5HT₃ ligand gated ion channel
- Sequence 6 Amino acid sequence of the human $\alpha 7$ /murine 5HT₃ ligand gated ion channel
- 10 Sequence 7 GG443 PCR Primer
- Sequence 8 GG444 PCR Primer
- 15 Sequence 9 DNA coding sequence of the mutant human $\alpha 7$ ligand gated ion channel containing the T→P mutation at amino acid position 230
- Sequence 10 Amino acid sequence of the mutant human $\alpha 7$ ligand gated ion channel containing the T→P mutation at amino acid position 230
- 20 Sequence 11 DNA coding sequence of the mutant human $\alpha 7$ ligand gated ion channel containing the C→S mutation at amino acid position 241
- Sequence 12 Amino acid sequence of the mutant human $\alpha 7$ ligand gated ion channel containing the C→S mutation at amino acid position 241
- 25 Sequence 13 DNA coding sequence of the double mutant human $\alpha 7$ ligand gated ion channel containing the T→P mutation at amino acid position 230 and the C→S mutation at amino acid position 241
- 30 Sequence 14 Amino acid sequence of the double mutant human $\alpha 7$ ligand gated ion channel containing the T→P mutation at amino acid position 230 and the C→S mutation at amino acid position 241

SUMMARY OF THE INVENTION

35 The present invention addresses the need identified above in that it provides methods and compositions useful for inducing inward conducting cation channels and cell lines expressing said channels to preferentially conduct calcium. Said inward cation channels can be either voltage gated ion channels, ligand gated channels, or non-voltage non-ligand gated ion channels.

40 In one embodiment, the invention includes a special cell culture medium comprising a high concentration of calcium and a relatively low concentration of sodium. The special cell culture medium comprises calcium ions at a concentration of from about 2 to 10 mM, sodium ions at a concentration of from about 0 to 50 mM, a pH between about 7.0-7.5, potassium between about 0.1 –

45 30mM and a buffer compatible with mammalian cells. Because the ionic

composition of the medium is reduced by the reduction in sodium ion content typically supplied by isotonic concentrations of sodium chloride the isotonicity of the media is retained by the addition of an impermeant cation in an amount sufficient to maintain isotonic conditions.

5 In another embodiment the invention includes methods of treating cells in aqueous culture medium, where the treatment comprises changing the aqueous environment of the cells from their beginning state, where they may exist in any aqueous buffered solution designed to maintain living cells, to a special cell culture medium where the ionic conditions comprise: calcium ions at a concentration of
10 from about 2 to 10 mM, sodium ions from about 0 to 50 mM, pH from about 7.0 to 7.5 and impermeant cations in an amount sufficient to maintain isotonic conditions.

In another embodiment the invention includes methods of inducing cells that express either voltage gated, ligand gated or non-voltage non-ligand gated inward conducting cation channels to preferentially conduct calcium ions. This is known as
15 calcium conductance or calcium flux, comprising: incubating the cells in a special cell culture medium described above for a length of time from between 15 minutes to about 8 hours. The conductance can then be measured in a variety of ways. A few of which are described.

In another particularly preferred embodiment the invention includes methods
20 of inducing cells that express $\alpha 7 / 5HT_3$ chimeric receptors to preferentially conduct calcium ions comprising the step of incubating the cells in the above mentioned special cell culture media.

In another particularly preferred embodiment the invention includes methods of inducing cells that express a mutant $\alpha 7$ receptor to preferentially conduct
25 calcium ions comprising the step of incubating the cells in the above mentioned special cell culture media.

In another embodiment the invention provides a chimeric $\alpha 7 / 5-HT_3$ nucleic acid molecule encoding a heretofore unknown chimeric ligand gated ion channel and constructs and recombinant host cells incorporating the isolated nucleic acid
30 molecules; chimeric $\alpha 7 / 5-HT_3$ polypeptides encoded by the isolated nucleic acid molecule and methods of making and using all of the foregoing.

In yet another embodiment the invention provides heretofore unknown mutants of the human $\alpha 7$ nAChR ligand gated ion channel and constructs and

recombinant host cells incorporating the isolated nucleic acid molecules; mutant $\alpha 7$ nAChR polypeptides encoded by the isolated nucleic acid molecules and methods of making and using all of the foregoing.

5 SEQ ID NOS: 5, 6, 9, 10, 11, 12, 13 and 14 provides particular human/mouse chimeric polynucleotide and polypeptide sequences and mutant $\alpha 7$ nAChR polynucleotide and polypeptide sequences, and the invention includes within its scope other human and mouse allelic variants and conservative amino acid substitutions. The polynucleotide sequences are intended to encompass the well known degeneracy of the genetic code.

10 In yet other embodiment the invention provides a fluorescent ligand binding assay comprising: incubating cells with a fluorescent ligand capable of binding to cell surface receptors and measuring the fluorescence of cell bound ligand using FLIPR. The invention also describes assays for selective agonists, antagonists and modulators of the $\alpha 7$ nAChR.

15 **ADDITIONAL DETAILS OF THE INVENTION**

There are many calcium influx assays suitable for high throughput screening but there are no good high throughput assays to measure the influx of other cations. Therefore it is desirable to induce a cell line that expresses inward conducting cation channels normally conducting other cations to preferentially conduct calcium. The present invention provides a methods and compositions of adapting an inward

20 conducting cation channel to preferentially conduct calcium. Such inward conducting cation channels include voltage gated ion channels, ligand gated ionic channels, and non-voltage gated non-ligand gated ionic channels.

Voltage gated ionic channels may be described as ion channels which open in response to a change in the voltage across the membrane. Ligand gated ion channels

25 may be described as ion channels which open in response to the binding of a ligand to the channel protein. Non-voltage non-ligand gated ion channels may be described as channels which don not open in response to either voltage across the membrane or to ligand binding but that are regulated by covalent modifications by second messenger signaling pathways such as protein phosphorylation, or increases in channel gene

30 expression leading to increases in ion channel density. Such a condition may exist, for example, in epithelial cells such as kidney epithelium cells and white blood cells.

As used herein the term "5HT-3 receptor" is used interchangeably with "5HT ligand gated ion channel" As used herein the term " $\alpha 7$ receptor" and " $\alpha 7$ nAChR"

and “ $\alpha 7$ ligand gated ion channel” are all used interchangeably. The term “mutant $\alpha 7$ receptors”, “mutant $\alpha 7$ ligand gated ion channel” or mutant “ $\alpha 7$ AchR” refers any one of a number of specific mutant polynucleotide or polypeptide species described herein. When a specific mutation is desired it referred to by the SEQ ID NO of its
5 encoding nucleic acid, or by reference to the SEQ ID NO of the resultant predicted polypeptide product. By way of example, a cell line expressing a particular mutation might be referred to as cells expressing the polynucleotide sequence of SEQ ID NO: 13 or the polypeptide sequence of SEQ ID NO:14. As aid in understanding the reader is directed to the section entitled “Brief Description of the Sequence Listings”

10 **Special Cell Culture Medium**

The inventors provide an ionic environment that can be used with all of the ion channels described herein. The special cell culture medium provides a means of adapting ligand gated, voltage gated, and non-ligand gated non-voltage gated ion channels not normally conducting calcium to the conductance of calcium. The
15 special cell culture medium provides a means of adapting those channels normally conducting sodium, potassium or other ions to the conductance of calcium whether those channels be of the ligand gated, voltage gated, or non-ligand non voltage gated variety.

The inventors have addressed the task of inducing calcium flux or calcium
20 conductance or transmission of calcium ions in ion channels not normally preferentially transmitting calcium ions by providing special cell culture compositions comprising a high concentration of calcium and a relatively low concentration of sodium. The special cell culture medium comprises calcium ions at a concentration of from about 2 to 10 mM, sodium ions at a concentration of from
25 about 0 to 50 mM, a pH between about 7.0-7.5, potassium between about 0.1 – 30mM and a buffer compatible with mammalian cells. It is understood by one of skill in the art that a variety of salts may be used as a source of sodium ions including but not limited by the examples of NaCl, Na_2HPO_4 , NaH_2PO_4 and NaHCO_3 . It is understood by one of skill in the art that a variety of salts may be used as a source of
30 potassium ions including but not limited by the examples of KCl, K_2HPO_4 , KH_2PO_4 and KHCO_3 . It is understood by one of skill in the art that calcium ions may be supplied by a variety of salts including but not limited by the examples of

CaCl₂ and CaSO₄. In addition all of the above ions may be supplied by salts of organic compounds within the knowledge of one of skill in the art.

Because the ionic composition of the medium is reduced by the reduction in sodium ion content typically supplied by isotonic concentrations of sodium chloride the isotonicity of the media is retained by the addition of an impermeant cation in an amount sufficient to maintain isotonic conditions. In the context of the present invention, the term "isotonic" means having an osmolality that is within the range tolerated by the cell or a solution that has the same osmotic pressure as the interior of the cell. Usually this is in the range of about 285-315 mOsm/kg H₂O depending on the cell type and source, more preferably about 290-305, for most cell types this is about 300 mOsm/kg H₂O.

Impermeant cations are defined as organic cations too large to pass through the channel of interest. By way of example only, such cations may include N-methyl-D-glucamine, choline, tetraethylammonium (TEA), tetrathymethylammonium (TMA) and tetrapropylammonium (TPA) and Tris.

In one particular embodiment, the cell culture medium comprises CaCl₂ at about 4mM, MgSO₄ at about 0.8 mM, HEPES Buffer at about 20mM, Glucose at about 6 mM, NaCl at about 20mM, KCl at about 5mM and the impermeant cation N-methyl-D-glucamine at about 120mM.

It is understood by one skilled in the art that calcium flux or the transmission of calcium ions may be accessed by a number of well know methods. These include but are not limited by the measurement of voltage changes either directly or indirectly caused by the movement of calcium ions *ie.* measuring ionic flux or conductance. In addition the presence of calcium may be accessed by its interaction with a number of flourescent dyes well known in the art. These include but are not limited by the choices of Calcium Green and flou-3 and flou-4. It is understood that the fluoresent signal of the various dyes known in the art may be measured on FLIPR but also on other more conventional instrumentation including fluorimeters

The present invention also provides a $\alpha 7/5$ -HT₃ chimeric receptor and a novel mutant human $\alpha 7$ receptors encoded by isolated polynucleotides (e.g., DNA sequences and RNA transcripts, both sense and complementary antisense strands, both single and double-stranded, including splice variants thereof) encoding a human enzyme referred to herein as $\alpha 7/5$ -HT₃ chimera or mutant $\alpha 7$ receptor DNA. Polynucleotides of the

invention include cDNA, and DNA that has been chemically synthesized in whole or in part. "Synthesized" as used herein and understood in the art, refers to polynucleotides produced by purely chemical, as opposed to enzymatic, methods.

"Wholly" synthesized DNA sequences are therefore produced entirely by chemical means, and "partially" synthesized DNAs embrace those wherein only portions of the resulting DNA were produced by chemical means. "Isolated" as used herein and as understood in the art, whether referring to "isolated" polynucleotides or polypeptides, is taken to mean that it is uniquely created by the inventors, separated from the original cellular or genetic environment in which the polypeptide or nucleic acid is normally found. As used herein therefore, by way of example only, a transgenic animal or a recombinant cell line constructed with a polynucleotide of the invention, incorporates the "isolated" nucleic acid.

Allelic variants are modified forms of a wild type gene sequence, the modification resulting from recombination during chromosomal segregation or exposure to conditions which give rise to genetic mutation. Allelic variants, like wild type genes, are naturally occurring sequences (as opposed to non-naturally occurring variants which arise from in vitro manipulation).

A DNA sequence encoding a $\alpha 7/5$ -HT₃ polypeptide is set out in SEQ ID NO: 5. DNA sequences encoding the mutant $\alpha 7$ receptor polypeptides are set out in SEQ ID NO: 9, 11 and 13. One of skill in the art will readily appreciate that the preferred DNA of the invention comprises a double stranded molecule, for example the molecule having the sequence set forth in SEQ ID NO: 5, 9, 11 or 13 along with the complementary molecule (the "non-coding strand" or "complement") having a sequence deducible from the sequence of SEQ ID NO: 5, 9, 11, or 13 according to Watson-Crick base pairing rules for DNA. Also preferred are other polynucleotides encoding the $\alpha 7/5$ -HT₃ polypeptides or mutant polypeptides of SEQ ID NO: 6, 10, 12, or 14 which differ in sequence from the polynucleotides of SEQ ID NO: 5, 9, 11 or 13 by virtue of the well known degeneracy of the genetic code.

The polynucleotide sequence information provided by the invention makes possible large-scale expression of the encoded polypeptide by techniques well known and routinely practiced in the art.

Autonomously replicating recombinant expression constructs such as plasmid and viral DNA vectors incorporating polynucleotides of the invention are also

provided. Expression constructs wherein $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor -encoding polynucleotides are operatively linked to an endogenous or exogenous expression control DNA sequence and a transcription terminator are also provided. Expression control DNA sequences include promoters, enhancers, and operators, and are generally selected based on the expression systems in which the expression construct is to be utilized. Preferred promoter and enhancer sequences are generally selected for the ability to increase gene expression, while operator sequences are generally selected for the ability to regulate gene expression. Expression constructs of the invention may also include sequences encoding one or more selectable markers that permit identification of host cells bearing the construct. Expression constructs may also include sequences that facilitate, and preferably promote, homologous recombination in a host cell. Preferred constructs of the invention also include sequences necessary for replication in a host cell.

Expression constructs are preferably utilized for production of an encoded protein, but also may be utilized simply to amplify a $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor -encoding polynucleotide sequence.

According to another aspect of the invention, host cells are provided, including prokaryotic and eukaryotic cells, comprising a polynucleotide of the invention (or vector of the invention) in a manner which permits expression of the encoded $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor polypeptide. Polynucleotides of the invention may be introduced into the host cell as part of a circular plasmid, or as linear DNA comprising an isolated protein coding region or a viral vector. Methods for introducing DNA into the host cell well known and routinely practiced in the art include transformation, transfection, electroporation, nuclear injection, or fusion with carriers such as liposomes, micelles, ghost cells, and protoplasts. Expression systems of the invention include bacterial, yeast, fungal, plant, insect, invertebrate, and mammalian cells systems.

Host cells for expression of $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor polypeptides include prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of $\alpha 7/5$ -HT₃ chimera receptor and or a mutant $\alpha 7$ receptors include but are not limited to bacteria of the genera *Escherichia*, *Bacillus*, and *Salmonella*, as well as members of the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*.

The isolated nucleic acid molecules of the invention are preferably cloned into a vector designed for expression in eukaryotic cells, rather than into a vector designed for expression in prokaryotic cells. Eukaryotic cells are preferred for expression of genes obtained from higher eukaryotes because the signals for synthesis, processing, and secretion of these proteins are usually recognized, whereas this is often not true for prokaryotic hosts (Ausubel, *et al.*, ed., in Short Protocols in Molecular Biology, 2nd edition, John Wiley & Sons, publishers, pg.16-49, 1992.). Eukaryotic hosts may include, but are not limited to, the following: insect cells, African green monkey kidney cells (COS cells), Chinese hamster ovary cells (CHO cells), human 293 cells, human SH-EP1 cells and murine 3T3 fibroblasts.

Expression vectors for use in prokaryotic hosts generally comprise one or more phenotypic selectable marker genes. Such genes generally encode, *e.g.*, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement. A wide variety of such vectors are readily available from commercial sources. Examples include pSPORT vectors, pGEM vectors (Promega), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), and pQE vectors (Qiagen).

The $\alpha 7/5$ -HT₃ chimera receptor and the novel mutant human $\alpha 7$ receptor may also be expressed in yeast host cells from genera including *Saccharomyces*, *Pichia*, and *Kluveromyces*. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Yeast vectors will often contain an origin of replication sequence from a 2 micron yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle vectors) may also be used. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli*.

Insect host cell culture systems may also be used for the expression of human $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor II polypeptides. In a preferred embodiment, the $\alpha 7/5$ -HT₃ chimera receptor and the novel mutant human $\alpha 7$ receptor II polypeptides of the invention are expressed using a baculovirus expression system. Further information regarding the use of baculovirus systems for the expression of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

In another preferred embodiment, the $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor II polypeptide is expressed in mammalian host cells. Non-limiting examples of suitable mammalian cell lines include the COS-7 line of monkey kidney cells (Gluzman *et al.*, *Cell* 23:175 (1981)), Chinese hamster ovary (CHO) cells, and human 293 cells.

The choice of a suitable expression vector for expression of the human $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor II polypeptide of the invention will of course depend upon the specific host cell to be used, and is within the skill of the ordinary artisan. Examples of suitable expression vectors include pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). Expression vectors for use in mammalian host cells may include transcriptional and translational control sequences derived from viral genomes. Commonly used promoter sequences and enhancer sequences which may be used in the present invention include, but are not limited to, those derived from human cytomegalovirus (CMV), Adenovirus 2, Polyoma virus, and Simian virus 40 (SV40). Methods for the construction of mammalian expression vectors are disclosed, for example, in Okayama and Berg (*Mol. Cell. Biol.* 3:280 (1983)); Cosman *et al.* (*Mol. Immunol.* 23:935 (1986)); Cosman *et al.* (*Nature* 312:768 (1984)); EP-A-0367566; and WO 91/18982.

The invention also provides $\alpha 7/5$ -HT₃ chimera receptor or novel mutant human $\alpha 7$ receptor II polypeptides encoded by a polynucleotides of the invention. Presently preferred is $\alpha 7/5$ -HT₃ chimera polypeptide comprising the amino acid sequence set out in SEQ ID NO: 6 and a novel mutant human $\alpha 7$ receptor comprising the amino acid sequence set out in SEQ ID NO: 14

Polypeptides of the invention may be produced natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving host cells of the invention. Use of mammalian host cells is expected to provide for such post-translational modifications (e.g., glycosylation, truncation, lipidation, and phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Glycosylated and non-glycosylated form of $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor II are embraced.

The invention also embraces variant $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor polypeptides wherein the essential activity, including

pharmacology which accurately mimics that of the native $\alpha 7$ ligand gated ion channel receptor of the $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor II is maintained. Examples of such variants include insertion, deletions or substitutions. Insertional variants also include fusion proteins wherein the amino and/or carboxy
5 termini of the $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor is fused to another polypeptide. It is further envisioned that the although the polypeptides of the invention are disclosed as mature protein sequences in SEQ ID NOS: 6, 10, 12, and 14, which include a signal sequence necessary for insertion into the cell membrane, the invention also includes polypeptides with the signal sequence
10 removed. Figure 2 provides a sequence representing indicating that the mature protein of $\alpha 7$ AChR derived polypeptides including the mutant polypeptides and the chimeric polypeptide have 22 amino acids removed in the mature form.

In another aspect, the invention provides deletion variants wherein one or more amino acid residues in a $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human
15 $\alpha 7$ receptor polypeptide are removed. Deletions can be effected at one or both termini of the $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor polypeptide, or with removal of one or more residues within the $\alpha 7/5$ -HT₃ chimera receptor or the novel mutant human $\alpha 7$ receptor amino acid sequence.

In still another aspect, the invention provides substitution variants of $\alpha 7/5$ -HT₃
20 chimera receptor and the novel mutant human $\alpha 7$ receptor polypeptides. Substitution variants include those polypeptides wherein one or more amino acid residues of a $\alpha 7/5$ -HT₃ chimera receptor and the novel mutant human $\alpha 7$ receptor polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature, however, the invention embraces substitutions that are also
25 non-conservative. Conservative substitutions for this purpose may be defined as set out in Tables A, B, or C below.

Variant polypeptides include those wherein conservative substitutions have been introduced by modification of polynucleotides encoding polypeptides of the invention. Amino acids can be classified according to physical properties and
30 contribution to secondary and tertiary protein structure. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in Table A

(from WO 97/09433, page 10, published March 13, 1997 (PCT/GB96/02197, filed 9/6/96), immediately below.

Table A**Conservative Substitutions I**

| | SIDE CHAIN CHARACTERISTIC | AMINO ACID |
|----|------------------------------|----------------|
| | <u>Aliphatic</u> | |
| 10 | Non-polar | G A P I L V |
| | Polar - uncharged | C S T M N Q |
| | Polar - charged | D E |
| 15 | | K R |
| | Aromatic | H F W Y |
| | Other | N Q D E |

Alternatively, conservative amino acids can be grouped as described in Lehninger,
 20 [Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77] as
 set out in Table B, immediately below

Table B
Conservative Substitutions II

| | | | |
|----|-------------------|------------------------------|-------------------|
| 5 | SIDE CHAIN | CHARACTERISTIC | AMINO ACID |
| | | Non-polar (hydrophobic) | |
| | | A. Aliphatic: | A L I V P |
| 10 | | B. Aromatic: | F W |
| | | C. Sulfur-containing: | M |
| | | D. Borderline: | G |
| | | Uncharged-polar | |
| | | A. Hydroxyl: | S T Y |
| 15 | | B. Amides: | N Q |
| | | C. Sulfhydryl: | C |
| | | D. Borderline: | G |
| | | Positively Charged (Basic): | K R H |
| | | Negatively Charged (Acidic): | D E |
| 20 | | | |

As still an another alternative, exemplary conservative substitutions are set out in Table C, immediately below.

Table C
Conservative Substitutions III

| | <u>Original Residue</u> | <u>Exemplary Substitution</u> |
|----|-------------------------|-------------------------------|
| 5 | Ala (A) | Val, Leu, Ile |
| | Arg (R) | Lys, Gln, Asn |
| | Asn (N) | Gln, His, Lys, Arg |
| | Asp (D) | Glu |
| 10 | Cys (C) | Ser |
| | Gln (Q) | Asn |
| | Glu (E) | Asp |
| | His (H) | Asn, Gln, Lys, Arg |
| 15 | Ile (I) | Leu, Val, Met, Ala, Phe, |
| | Leu (L) | Ile, Val, Met, Ala, Phe |
| | Lys (K) | Arg, Gln, Asn |
| | Met (M) | Leu, Phe, Ile |
| | Phe (F) | Leu, Val, Ile, Ala |
| 20 | Pro (P) | Gly |
| | Ser (S) | Thr |
| | Thr (T) | Ser |
| | Trp (W) | Tyr |
| | Tyr (Y) | Trp, Phe, Thr, Ser |
| 25 | Val (V) | Ile, Leu, Met, Phe, Ala |

Example 1

Construction of chimeric $\alpha 7/5$ -HT₃ receptor

PCR Primers GG443 (SEQ ID NO:7) and GG444 SEQ ID NO:8 were used to isolate
 30 the DNA encoding the N-terminal 201 amino acids from the human $\alpha 7$ nAChR
 (Fig1).

GG443: 5' GGCTCTAGACCACCATGCGCTGTTACCGGGAGGCGTCTGGCTG 3'

GG444: 5' GGGTGATCACTGTGAAGGTGACATCAGGGTAGGGCTC 3'

The isolated DNA fragment of encoding the N-terminus of the $\alpha 7$ was engineered to have an Xba 1 site at the 5' end and Bcl 1 site at the 3' end. The engineered restriction sites are underlined in each respective primer. The pore forming domain of the mouse 5-HT₃ cDNA was then isolated as a Bcl 1/Sal 1 DNA fragment of the complete mouse cDNA gene. A ligation reaction was used to join the 5' of the $\alpha 7$ cDNA with the 3' end of the 5-HT₃ cDNA. This ligated fragment was isolated and purified and then cloned into the Xba1 Sal 1 site of two mammalian expression plasmid vectors termed pGG764 and pGG759. The parental plasmid termed pGG764, which contained the G418 resistance gene also contained a cytomegalovirus (CMV) promoter and a bovine growth hormone polyadenylation site for the initiation and termination of mRNA transcription. The parental plasmid termed pGG759 contained the hygromycin resistance gene and the identical mRNA initiation and termination regulatory elements. The new plasmid derived from the insertion of $\alpha 7/5$ -HT₃ gene into pGG764 was termed pGS175. The new plasmid derived from the insertion of $\alpha 7/5$ -HT₃ gene into pGG759 was termed pGS176. Both pGS175 and pGS179 were transformed into E. coli and isolated colonies were picked and expanded. The DNA from each plasmid was isolated and sequenced to verify that both constructions were correct. The sequence obtained for the coding region of the $\alpha 7/5$ -HT₃ cDNA construct is shown in SEQ ID NO: 5 and the predicted amino acid sequence of the construct is given in in SEQ ID NO: 6

It is understood that once one skilled in the art has possession of applicant's chimeric $\alpha 7/5$ -HT₃ and mutant $\alpha 7$ AChRs a number of novel assays are evident for the assessment of ligand binding, of the ability of test compounds to function as agonists, and to measure the ability of test compounds to function as modulators of $\alpha 7$ activity. Details are provided in the examples below. It is understood however that one skilled in the art might perform the same essential functions in a variety of way and the examples are in no way intended to indicate limitations in the claims.

Expression of the Chimeric Receptor

The $\alpha 7/5$ -HT₃ cDNA inserted into pGS175 and pGS179 were simultaneously transfected into SH-EP1 cells using cationic lipid transfection reagent and cells expressing the $\alpha 7/5$ -HT₃ channel were selected using 800 μ g/ml geneticin (G418) and 400 μ g/ml of hygromycin B. Cells expressing the chimeric protein at high levels were identified by measuring fluorescein- α -bungarotoxin binding (see Figure 3) . Isolated

clones were grown in Eagle's minimal essential medium (MEM) supplemented with 10 % fetal bovine serum (FBS), 4 mM L-Glutamine, Fungi-Bact.(1:100), 400 µg/ml hygromycin B, and 800 µg/ml G418. All cells were maintained in an incubator at 37°C in a humidified 6 % CO₂ atmosphere.

5

Example 2

Fluorescein labeled α-bungarotoxin (fl-btx) binding assay

The α7/5-HT₃-SHEP cells were grown in minimal essential medium (MEM) containing nonessential amino acids supplemented with 10 % fetal bovine serum, L-glutamine, 100 units/ml penicillin/streptomycin, 250 ng/ml fungizone, 400 µg/ml Hygromycin-B, and 800 µg/ml Geneticin. The cells were grown in a 37°C incubator with 6 % CO₂. The α7/5-HT₃-SHEP cells were trypsinized and plated in 96 well plates with dark side walls and clear bottoms (Corning # 3614) at density of 2.6 X 10⁴ cells per well two days before analysis. On the day of the analysis, the cells were washed four times using a Bio-Tek plate washer. After the fourth cycle, the final volume in each well was 100 µl. Cellular fluorescence was analyzed on FLIPR (Molecular Devices) after the addition of a 100 µl of a 2X stock fluorescein labeled α-bungarotoxin (F-1176 Molecular Probes: Fl-btx). In competition experiments the competing ligand was added as a 2x drug stock before the addition of Fl-btx. Fluorescence was measured by exciting the dye at 488 nm using 500 mW of power. A 0.5 second exposure was used to illuminate each well. Fluorescence emission was recorded above 525 nm. Fluorescence was detected using a F-stop set of either 2.0 or 1.2. The cellular fluorescence was so intense that subsequent washing was not needed to measure cellular fluorescence.

The data in Figure 3 shows that Fl-btx binding is a saturable reaction with a K_i of 15.5 nM. Nicotine at 100 µM competes at all concentrations of Fl-btx (Figure 3). Figure 4 and 5 show that epibatidine and unlabeled α-btx also compete for Fl-btx binding with a K_i of 90 nM and 33 nM respectively. The data in Table 1 provide a summary of the effect of seven structurally unrelated molecules in the whole cell Fl-btx binding assay.

30

| Agonists/Antagonists | Fltc- α -Bungarotoxin Binding (30nM) |
|--------------------------|---|
| (-) Nicotine | IC ₅₀ = 9.7 μ M |
| (+/-) Epibatidine | IC ₅₀ = 90 nM |
| GTS-21 | IC ₅₀ = 16 μ M |
| ABT-418 | IC ₅₀ = 38 μ M |
| Anabasiene | IC ₅₀ = ND |
| Mecamylamine | IC ₅₀ = >300 μ M |
| Methyllycaconitine (MLA) | IC ₅₀ = 26 nM |

The rank order potency of these compounds follow the known pharmacology of $\alpha 7$ nAChR (Holliday et al 1997). Taken together these data show that the fl-btx binding assay on the $\alpha 7/5$ -HT₃ chimera receptor can be used to novel and selective agonists and antagonists of endogenous $\alpha 7$ nAChR.

The whole cell binding assay described in this example is useful in many regards not the least of which is that $\alpha 7$ nAChR is in its native configuration, only cell surface $\alpha 7$ nAChR is a binding target, the assay is simpler because there is no need to prepare membranes, and there are no radioisotopes being used and because fluorescence is detected within approximately 200 microns of the bottoms of the wells the need for extensive washing is eliminated.

Our results as summarized in the Figures demonstrate that the $\alpha 7/5$ -HT₃ SH-EP cell line can be used in the Fl-btx binding assay on FLIPR. The pharmacology of the $\alpha 7/5$ -HT₃ receptor suggests that the Fl-btx binding assay can be used in a HTS format to find novel $\alpha 7$ nAChR agonists and antagonists.

Example 3

Calcium Flux Assay—Identification of an $\alpha 7$ nAChR Agonist

The $\alpha 7/5$ -HT₃-SHEP or alternatively the human $\alpha 7$ nAChR double mutant SHEP (described below) cells were grown in minimal essential medium (MEM) containing nonessential amino acids supplemented with 10 % fetal bovine serum, L-glutamine, 100 units/ml penicillin/streptomycin, 250 ng/ml fungizone, 400 μ g/ml Hygromycin-B, and 800 μ g/ml Geneticin. The cells were grown in a 37⁰ C incubator with 6 % CO₂. The $\alpha 7/5$ -HT₃-SHEP cells were trypsinized and plated in 96 well plates with dark side walls and clear bottoms (Corning # 3614) at density of 2.6 X 10⁴ cells per well two

days before analysis. The cells were loaded in a 1:1 mixture of 2 mM Calcium Green-1, AM (Molecular Probes) prepared in anhydrous dimethylsulfoxide and 20 % pluonic F-127 (Molecular Probes). This reagent was added directly to the growth medium of each well to achieve a final concentration of 2 μ M of Calcium Green-1, AM. The cells
5 were incubated in the dye for one hour at 37⁰ C and then washed with 4 cycles of Bio-Tek plate washer. Each cycle was programmed to wash each well with four times with either EBSS or MMEBSS. After the third cycle, the cells were allowed to incubate at 37⁰ C for at least ten minutes. After the fourth cycle final volume in each well was 100 μ l. The cells were analyzed on FLIPR (Molecular Devices) for the change in
10 fluorescence after the addition of a 100 μ l of a 2X drug stock. FLIPR was set up to excite the dye with at 488 nanometers using 500 mW of power. A 0.5 second exposure was used to illuminate each well. Fluorescence emission was recorded above 525nm. Fluorescence was detected using a F-stop set of either 2.0 or 1.2.

Under physiological ionic conditions, the 5-HT₃ ligand gated ion channel
15 conducts primarily Na⁺ and is a poor conductor of Ca⁺⁺ (Yang 1990; Brown et al 1998). Whereas, under physiological ionic conditions the α 7 nACh channel conducts primarily Ca⁺⁺.

Therefore a particular embodiment of a special cell culture media, designated MMEBSS was used to enhance the agonist-evoked flux of calcium through the α 7/5-HT₃ channel expressed in SH-EP1 cells (Fig 6). We compared the physiological
20 Earles Balanced Salt Solution (EBBS) buffer and the special cell culture media (MMEBSS) in the Ca⁺⁺ functional assay on FLIPR. The result of this experiment clearly indicated that under physiological conditions (EBBS) little calcium was detected in response to a maximally effective concentration of (-) nicotine (100 μ M).
25 Other the other hand using the special cell culture media, (MMEBSS) 100 μ M (-) nicotine evoked a large increase in intracellular calcium (Figure 6). Under these conditions, FLIPR can be used to accurately measure agonist activity of the α 7/5-HT₃ channel (Table 2). The α 7/5-HT₃-SH-EP1 cells express an endogenous bradykinin receptor that when stimulated with 100 nM bradykinin produces a maximal increase
30 in intracellular calcium by releasing calcium from intracellular stores. The data in Figure 7 show that the bradykinin-induced calcium flux was similar in EBSS and MMEBSS. These data indicate that the effect of MMEBSS was specific for the calcium flux through the α 7/5-HT₃ channel

- The special cell culture media, designated MMEBSS is comprised of 4 mM CaCl_2 , 0.8 mM MgSO_4 , 20 mM NaCl , 5.3 mM KCl , 5.6 mM D-Glucose, 120 mM N-Methyl-D-Glucosamine, 9 mM Tris base and 20 mM HEPES. A detailed description of the preparation of MMEBSS is provided below. It should be recognized however
- 5 that the recipe below is provided by way of example only and that the applicants intends to claim the full range of what they have invented.

| MMEBSS Buffer | | | |
|--|----------------|----------|---------------------|
| Buffer Component | Stock Solution | 2 Liters | Final Concentration |
| CaCl_2 Dihydrate | 1M | 10 ml. | 4 mM |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 1M | 1.6 ml. | 0.8 mM |
| NaCl | 2M | 20 ml. | 20 mM |
| KCl | | 0.8 gr. | 5.3 mM |
| D-Glucose | | 2.0 gr. | 5.6 mM |
| Tris-HEPES ¹ | 1M | 40 ml. | 20 mM |
| N-Methyl-D-Glucamine (pH 7.3) ² | 1.36 | 176.5 ml | 120 mM |
| Tris Base ³ | | 0.5 gr | |

¹ 1M. Tris – HEPES pH 7.4 is formulated by weighing 47.66 grams of HEPES and adding approximately 8 of Tris base in 150 ml of water, the pH is adjusted to 7.4 with HCl. The final volume is adjusted to 200 ml.

- 10 ² 1.36 M. N-Methyl – D – Glucamine /HCl pH 7.3 is formulated by adding 265.47 grams of N-Methyl-D-Glucamine in 500 ml. water 115 ml concentrated HCl is then added to the solution with stirring. The final pH is adjusted to 7.4

³ Final concentration of Tris in buffer is approximately 9mM

For the experiments described above the physiologic buffer designated Earles

- 15 Balanced Salt Solution was also prepared or purchased.

The compositions of EBSS and MMEBSS are compared below.

| Earle's Balanced Salt Solution (EBSS) | |
|--|----------|
| CaCl_2 | 1.8 mM |
| MgSO_4 | 0.8 mM |
| NaHPO_4 | 1.0 mM |
| NaHCO_3 | 26 mM |
| Hepes | 20 mM |
| Glucose | 5.6 mM |
| NaCl | 117.0 mM |
| KCl | 5.3 mM |

| MMEBSS | |
|--------------------------|-----------------------------------|
| CaCl₂ | 4.0 mM |
| MgSO ₄ | 0.8 mM |
| NaHPO₄ | 0.0 mM |
| NaHCO₃ | 0.0 mM |
| Hepes | 20 mM |
| Glucose | 5.6 mM |
| NaCl | 20.0 mM |
| KCl | 5.3 mM |
| N-methyl-D-glucamine | 120 mM also includes Tris base |

The summary of the pharmacological results using the $\alpha 7/5$ -HT₃ channel as a drug target is listed in Table 2.

| Characterization of the $\alpha 7/5$ -HT ₃ Chimeric Channel | | |
|--|---|--|
| | $\alpha 7/5$ -HT ₃ EC ₅₀ (μ M)* | $\alpha 7$ nAChR EC ₅₀ (μ M)* |
| Nicotine | 5.7 | 10-50 |
| Epibatidine | 0.120 | 2 |
| ABT | 27 | 70 |
| Anabaseine | 6.6 | 6 |
| GTS-21 | 30 | 30 |

*"EC₅₀" is the effective concentration that produces a 50% maximal response.

These data establish that agonist activity of the $\alpha 7/5$ -HT₃ channel can be used to predict the agonist activity at the endogenous $\alpha 7$ nACh receptor and thus provide evidence that the $\alpha 7/5$ -HT₃ channel can be use as a drug target to find novel $\alpha 7$ nAChR agonists.

Example 4

Calcium Flux Assay---Identification of an $\alpha 7$ nAChR Antagonist

The SH-EP1 cells expressing the $7/5$ -HT₃ nAChR ($7/5$ -HT₃ -SHEP) or alternatively the human $\alpha 7$ nAChR double mutant SHEP cells(described below) were grown in minimal essential medium (MEM) containing nonessential amino acids supplemented with 10 % fetal bovine serum, L-glutamine, 100 units/ml penicillin/streptomycin, 250 ng/ml fungizone, 400 ug/ml Hygromycin-B, and 800 ug/ml Geneticin. The cells were grown in a 37⁰ C incubator with 6 % CO₂. The $7/5$ -HT₃ -SHEP cells were trypsinized and plated in 96 well plates with dark side walls and clear bottoms (Corning # 3614) at density of 2.6 X 10⁴ cells per well two days before analysis. The $7/5$ -HT₃ -SHEP cells were loaded in a 1:1 mixture of 2 mM Calcium Green-1, AM

(Molecular Probes) prepared in anhydrous dimethylsulfoxide and 20 % pluonic F-127 (Molecular Probes). This reagent was added directly to the growth medium of each well to achieve a final concentration of 2 M of Calcium Green-1, AM. The $\alpha 7/5$ -HT₃-SHEP cells were incubated in the dye for one hour at 37⁰ C and then washed with 4 cycles of Bio-Tek plate washer. Each cycle was programmed to wash each well with four times with either EBSS or MMEBSS. After the third cycle, the $\alpha 7/5$ -HT₃-SHEP cells were allowed to incubate at 37⁰ C for at least ten minutes. After the fourth cycle final volume in each well was 100 μ l. Antagonist activity was measured as a decrease in nicotine-induced calcium influx using $\alpha 7/5$ -HT₃ channel as a drug target. FLIPR (Molecular Devices) was set up to measure intracellular calcium by exciting the Calcium Green with at 488 nanometer using 500 mW of power and reading fluorescence emission above 525 nanometers. A 0.5 second exposure was used to illuminate each well. Fluorescence was detected using a F-stop set of either 2.0 or 1.2. Specifically, after 30 seconds of baseline recording, test compounds were added to each well of a 96 well plate using 50 μ l from a 3X drug stock. 180 seconds after the addition of the test compounds, nicotine was added to each well to achieve a final concentration of 100 μ M. In each experiment, 4 wells were used as solvent controls. As indicated in Figure 8 antagonist activity was measured as a decrease in the 100 μ M nicotine-induced calcium influx relative to the effect of 100 μ M nicotine in the solvent control wells.

Example 5

Construction of the human $\alpha 7$ mutant receptors

We discovered that it was possible by introducing certain non-conservative amino acid changes at the amino acid positions corresponding to positions 230 and 241 of the human sequence to recreate the desirable properties of the human/mouse $\alpha 7$ nAChR/5-HT₃ hybrid.

The two primer system utilized in the Transformer Site-Directed Mutagenesis kit from Clontech (LaJolla CA), may be employed for introducing site-directed mutants into the human $\alpha 7$ sequence of SEQ ID NO: 1. Following denaturation of the target plasmid in this system, two primers are simultaneously annealed to the plasmid; one of these primers contains the desired site-directed mutation, the other contains a mutation at another point in the plasmid resulting in elimination of a restriction site. Second strand synthesis is then carried out, tightly linking

these two mutations, and the resulting plasmids are transformed into a mutS strain of *E. coli*. Plasmid DNA is isolated from the transformed bacteria, restricted with the relevant restriction enzyme (thereby linearizing the unmutated plasmids), and then retransformed into *E. coli*. This system allows for
5 generation of mutations directly in an expression plasmid, without the necessity of subcloning or generation of single-stranded phagemids. The tight linkage of the two mutations and the subsequent linearization of unmutated plasmids results in high mutation efficiency and allows minimal screening. Following synthesis of the initial restriction site primer, this method requires the use of only one
10 new primer type per mutation site. Transformants can be screened by sequencing the plasmid DNA through the mutagenized region to identify and sort mutant clones. Each mutant DNA can then be fully sequenced or restricted and analyzed by electrophoresis on Mutation Detection Enhancement gel (J. T. Baker) to confirm that no other alterations in the sequence have occurred (by band shift comparison to
15 the unmutagenized control).

A mutant $\alpha 7$ is prepared using Transformer TM site-directed mutagenesis kit, according to the manufacturer's protocol roughly outlined above. In one mutant, a codon in the channel mRNA is changed from ACG to CCG with the A at position 688 being changed to a C thus creating a mutant channel with threonine changed to
20 proline at amino acid position number 230. The polynucleotide and predicted amino acid sequence of the entire mutant $\alpha 7$ ligand gated ion channel containing the T→P mutation is set forth in SEQ ID NO: 9 and 10 respectively. In another mutant, a codon in the channel mRNA is changed from TGT to AGT with the T at position 721 being changed to A thus creating a mutant channel with cysteine changed to
25 serine at amino acid position 241. The polynucleotide and predicted amino acid sequence of the entire mutant $\alpha 7$ ligand gated ion channel containing the C→S mutation is set forth in SEQ ID NO: 11 and 12 respectively. In another mutant, both of the above mentioned mutations are introduced into the same DNA construct encoding a channel mRNA. The polynucleotide and predicted amino acid sequence
30 of the double mutant $\alpha 7$ ligand gated ion channel containing the T→P mutation and the C→S mutation is set forth in SEQ ID NO: 13 and 14 respectively.

This double mutant channel protein has been shown to exhibit the desirable characteristics of the chimeric $\alpha 7/5$ -HT₃ ligand gated ion channel including stability

and assay characteristics when expressed in human SH-EP1 cells. Exemplary expression methods are described elsewhere and are fully within the ordinary skill of one in the art.

Example 6

5 Functional Results with Double Mutant

The SH-EP1 cells expressing the double mutation of SEQ ID NO:13 (double mutant SHEP cells)are grown in minimal essential medium (MEM) containing nonessential amino acids supplemented with 10 % fetal bovine serum, L-glutamine, 100 units/ml penicillin/streptomycin, 250 ng/ml fungizone, 400 ug/ml Hygromycin-B, and 800
10 ug/ml Geneticin. The cells are grown in a 37⁰ C incubator with 6 % CO₂. The 7- double mutant SHEP cells were trypsinized and plated in 96 well plates with dark side walls and clear bottoms (Corning # 3614) at density of 2.6 X 10⁴ cells per well two days before analysis. The double mutant-SHEP cells are loaded in a 1:1 mixture of 2 mM Calcium Green-1, AM (Molecular Probes) prepared in anhydrous
15 dimethylsulfoxide and 20 % pluonic F-127 (Molecular Probes). This reagent was added directly to the growth medium of each well to achieve a final concentration of 2 M of Calcium Green-1, AM. The double mutant SHEP cells were incubated in the dye for one hour at 37⁰ C and then washed with 4 cycles of Bio-Tek plate washer. Each cycle was programmed to wash each well with four times with either EBSS or
20 MMEBSS. After the third cycle, the double mutant-SHEP cells were allowed to incubate at 37⁰ C for at least ten minutes. After the fourth cycle final volume in each well was 100 l. Expression of the mutant $\alpha 7$ receptor was analyzed by measuring agonist-induced changes in intracellular calcium accumulation. FLIPR (Molecular Devices) was set up to excite Calcium Green with at 488 nanometer using 500 mW
25 of power and reading fluorescence emission above 525 nanometers. A 0.5 second exposure was used to illuminate each well. Fluorescence was detected using a F-stop set of either 2.0 or 1.2. Specifically, after 30 seconds of baseline recording, test compounds were added to each well of a 96 sell plate using a 100 l from a 2X drug stock. In each experiment, at least 4 wells contained 7/5-HT₃-SHEP cells as positive
30 controls. As indicated in Figure 9 agonist activity was measured as an increase in intracellular calcium over baseline. As indicated in Figure 9 this paradigm identified clonal cell lines that functionally expressed the double mutant $\alpha 7$ receptor. All

attempts to express the wild type 7 nAChR using similar methods were totally unsuccessful.

Example 7

Calcium Flux Assay: Modulator Screen

5

The SH-EP1 cells expressing the double mutation of SEQ ID NO:13 (double mutant SHEP cells) were grown in minimal essential medium (MEM) containing nonessential amino acids supplemented with 10 % fetal bovine serum, L-glutamine, 100 units/ml penicillin/streptomycin, 250 ng/ml fungizone, 400 g/ml Hygromycin-

10

B, and 800 ug/ml Geneticin. The cells were grown in a 37⁰ C incubator with 6 % CO₂. The cells were trypsinized and plated in 96 well plates with dark side walls and clear bottoms (Corning # 3614) at density of 2.6×10^4 cells per well two days before analysis. The double mutant SHEP cells were loaded in a 1:1 mixture of 2 mM Calcium Green-1, AM (Molecular Probes) prepared in anhydrous dimethylsulfoxide and 20 % pluronic F-127 (Molecular Probes). This reagent was added directly to the growth medium of each well to achieve a final concentration of 2 M of Calcium Green-1, AM. The double mutant SHEP cells were incubated in the dye for one hour at 37⁰ C and then washed with 4 cycles of Bio-Tek plate washer. Each cycle was programmed to wash each well with four times with either EBSS or MMEBSS.

20

After the third cycle, the double mutant-SHEP cells were allowed to incubate at 37⁰ C for at least ten minutes. After the fourth cycle final volume in each well was 100 ul. Allosteric modulator activity was measured as the drug dependent increase in the agonist activity using the double mutant AChR channel as a drug target. Modulator induce increase in agonist activity was measured by increasing intracellular calcium accumulation. FLIPR (Molecular Devices) was set up to excite Calcium Green with at 488 nanometer using 500 mW of power and reading fluorescence emission above 525 nanometers. A 0.5 second exposure was used to illuminate each well.

25

Fluorescence was detected using a F-stop set of either 2.0 or 1.2. Specifically, after 30 seconds of baseline recording, test compounds were added to each well of a 96 well plate using a 50 l from a 3X drug stock. In each experiment, 4 wells were used as solvent controls. As indicated in Figure 10 modulator activity produced an increase in the nicotine-induced influx of intracellular calcium. The preferred

30

modulator had no effect in the absence of agonist. All data is plotted relative to the effect of 100 M nicotine, which induced a maximal calcium influx.

Example 8

Changing the ionic conditions of cellular medium is also likely to increase the calcium influx on many other ion channels that do not conduct calcium under physiological conditions. For example, it is known that the P2X(2) family of purinoceptors are cation-selective channels that are activated by ATP and its analogues. The ionic selectivity of this channel is $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+ >>> Ca^{++}$. In addition, divalent ions such induce a block of the channel that is measured by a reduction in amplitude of the unitary currents. Organic cations such as NMDG(+), Tris(+), TMA(+) and TEA(+) are virtually impermeant. It is likely that the ionic composition of MMEBSS will establish conditions that will permit Ca^{++} ions to pass through the channel in sufficient quantities to use a calcium influx assay to measure channel activity. Under these conditions, a calcium influx assay can be used as a high throughput assay using P2X receptors as a drug target.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art, all of which are intended as aspects of the invention. Accordingly only such limitations as appear in the claims should be placed in the invention.

Claims

What is claimed is:

1. A special cell culture medium comprising:
 - 5 a) calcium ions at a concentration of from 2 to 10 mM,
 - b) sodium ions at a concentration of from 0 to 50 mM,
 - c) pH between about 7.0- 7.5,
 - d) impermeant organic cations at a concentration sufficient to make the solution isotonic,
 - 10 e) K^+ ions between about 0.1 - 30 mM, and
 - f) a buffer compatible with mammalian cells.
2. The special cell culture medium of claim 1 where;
 - 15 a) calcium ions are at a concentration of from about 2 to 8 mM,
 - b) sodium ions at a concentration of from about 15 to 40 mM,
 - c) pH is between about 7.2- 7.4,
 - d) impermeant organic cations at a concentration sufficient to make the solution isotonic, where the impermeant organic cations are selected from the group consisting of N-methyl-D-glucamine, N-methyl-D-glucamine plus Trizma base,
 - 20 choline, TEA, TMA and TPA, and
 - e) K^+ ions between about 2.0 - 6.0 mM .
3. The special cell culture medium of claim 2 where:
 - 25 a) calcium ions are at a concentration of from about 4 to 8 mM,
 - b) sodium ions at a concentration of from about 15 to 25 mM,
 - c) pH is between about 7.2- 7.4, and
 - e) K^+ ions between about 4.0 - 5.0 mM.
4. The special cell culture medium of claim 3 where:
 - 30 a) calcium ions are at a concentration of about 4 mM,
 - b) sodium ions at a concentration of about 20 mM,
 - c) pH is about 7.4,

d) impermeant organic cations at a about 300 mOsm/kg H₂O, where the impermeant organic cations N-methyl-D-glucamine,

e) K⁺ ions are about 5.3 mM and provided from KCl.

5 5. The special cell culture of claim 1 (PCT 1-4) which also contains a nutrient source, except the nutrient source cannot be lactate unless the cells are liver cells.

6. The special cell culture of claim 5 (PCT 1-5) where the nutrient source is glucose, glutamine, pyruvate but not lactate unless the cells are liver cells and where
10 the f) buffer compatible with mammalian cells is HEPES.

7. The special cell culture of claim 6 that is MMEBSS.

8. A method of treating cells in aqueous culture medium, where the treatment
15 comprises changing the aqueous environment of the cells from their beginning state, where they may exist in any aqueous buffered solution designed to maintain living cells to an special cell culture medium where the ionic conditions comprise:

- a) calcium ions at a concentration of from about 2 to 10 mM,
- b) sodium ions at a concentration of from about 0 to 50 mM,
- 20 c) pH is between about 7.0- 7.5,
- d) impermeant organic cations at a concentration sufficient to make the solution isotonic.

9. The method of claim 9 where;

- 25 a) calcium ions are at a concentration of from about 2-8 mM,
- b) sodium ions at a concentration of from about 15 to 40 mM,
- c) pH is between about 7.2- 7.4,
- d) impermeant organic cations at a concentration sufficient to make the solution isotonic, where the impermeant organic cations are selected from the group
30 consisting of N-methyl-D-glucamine, , choline, TEA, Tris TMA and TPA.

10. The method of claim 9 where:

- a) calcium ions are at a concentration of from about 4 to 8 mM,

- b) sodium ions at a concentration of from about 15 to 25 mM,
- c) pH is between about 7.2- 7.4.

- 5 11. The method of claim 10 where:
- a) calcium ions are at a concentration of about 4 mM,
 - b) sodium ions at a concentration of about 20 mM,
 - c) pH is about 7.4,
 - d) impermeant organic cations at about 300 mOsm/kg H₂O, where the
- 10 impermeant organic cations N-methyl-D-glucamine.

12. The method of claim 11 (PCT 7-10) which also contains a nutrient source, except the nutrient source cannot be lactate unless the cells are liver cells.

- 15 13. The method of claim 12 (PCT 7-11) where the nutrient source is glucose, glutamine, pyruvate but not lactate unless the cells are liver cells.

14. The method of claim 8 where said aqueous environment of the cells from their beginning state is an Earle's Balanced Salt Solution or EBSS.

20

15. A method of inducing a mammalian cell line that expresses: voltage gated, ligand gated or non-voltage non-ligand gated inward conducting cation channels to preferentially conduct calcium ions, known as calcium flux, comprising: incubating the cells in a special cell culture medium comprising:

- 25
- a) calcium ions at a concentration of from 2 to 10 mM,
 - b) sodium ions at a concentration of from 0 to 50 mM,
 - c) pH is between 7.0- 7.5
 - d) impermeant organic cations at a concentration sufficient to make the solution isotonic,
- 30
- e) K⁺ ions between about 0.1 - 30 mM, and
 - f) a buffer compatible with mammalian cells,

for a length of time from about 15 minutes to about 8 hours.

16. The method of claim 15 where said mammalian cell line that expresses a voltage gated, ligand gated or non-voltage non-ligand gated inward conducting cation channels, is further described as expressing a non-selective channel, a sodium
5 channel, a potassium channel or a calcium channel.

17. The method of claim 16 where said mammalian cell line expresses a voltage gated, inward conducting cation channel that expresses a non-selective channel, a sodium channel, a potassium channel or a calcium channel.
10

18. The method of claim 16 where said mammalian cell line expresses a ligand gated inward conducting cation channels that expresses a non-selective channel, a sodium channel, a potassium channel or a calcium channel.

15 19. The method of claim 17 where said mammalian cell line expresses a non-voltage non-ligand gated inward conducting cation channel that expresses a non-selective channel, a sodium channel, a potassium channel or a calcium channel.

20. The method of claim 17 where said channel is a non-selective channel.
20

21. The method of claim 17 where said channel is a sodium channel.

22. The method of claim 17 where said channel is a potassium channel.

25 23. The method of claim 18 where said channel is a non-selective channel.

24. The method of claim 18 where said channel is a sodium channel.

25. The method of claim 18 where said channel is a potassium channel.
30

26. The method of claim 19 where said channel is a non-selective channel.

27. The method of claim 19 where said channel is a sodium channel.

28. The method of claim 19 where said channel is a potassium channel.
29. The method of claim 14 where said buffer compatible with mammalian cells is
5 the buffer known as HEPES and the impermeant organic cations are at about 300
mOsm/kg H₂O, where the impermeant organic cations are selected from N-methyl-D-
glucamine, N-methyl-D-glucamine, Tris, choline, TEA, TMA and TPA and the K⁺
ions are provided from KCl.
- 10 30. The method of claim 8 where said channel has an amino acid sequence
comprising residues 23-470 of SEQ ID NO:6
31. The method of claim 8 where said channel has an amino acid sequence
15 comprising residues 23- 502 of SEQ ID NO:14
32. The method of claim 8 where said channel is a 5-HT₃ channel
33. The method of claim 15 where said channel has an amino acid sequence
20 comprising residues 23-470 of SEQ ID NO:6
34. The method of claim 15 where said channel has an amino acid sequence
comprising residues 23- 502 of SEQ ID NO:14
- 25 35. The method of claim 15 where said channel is a 5-HT₃ channel.
36. A method of measuring the calcium flux of mammalian cells comprising:
a) growing the cells in an aqueous cell culture medium,
b) treating the cells using the treatments described in claim 7 (PCT 7-12),
30 c) measuring the calcium conductance.
37. The method of claim 36 where said calcium conductance is measured using
fluorescent dyes.
- 35 38. The method of claim 37 where said fluorescent dyes are used with the FLIPR
calcium flux assay system.

39. The method of claim 38 where said calcium conductance is measured by measuring the ionic flux of said conductance.
- 5 40. The method of claim 39 where said aqueous cell culture medium is Earle's Balanced Salt Solution or EBSS.
- 10 41. An isolated nucleic acid comprising the nucleotide sequence that encodes the amino acid sequence of residues 23 through 470 of SEQ ID NO:6
42. An isolated nucleic acid comprising the nucleotide sequence that encodes the amino acid sequence of SEQ ID NO:6
- 15 43. The isolated nucleic acid of claim 41 which has the nucleotide sequence between positions 67 through 1416 of SEQ ID NO:5
44. The isolated nucleic acid of claim 42 which has the nucleotide sequence of SEQ ID NO:5
- 20 45. A vector comprising the nucleic acid of Claim 41.
46. A vector comprising the nucleic acid of Claim 41 operably linked to an expression control sequence.
- 25 47. A host cell comprising the vector of claim 46
48. An isolated nucleic acid comprising the nucleotide sequence that encodes the amino acid sequence of residues 23 through 502 of SEQ ID NO:10
- 30 49. An isolated nucleic acid comprising the nucleotide sequence that encodes the amino acid sequence of SEQ ID NO:10
50. The isolated nucleic acid of claim 48 which has the nucleotide sequence between positions 67 through 1509 of SEQ ID NO:9
- 35 51. The isolated nucleic acid of claim 49 which has the nucleotide sequence of SEQ ID NO:9
52. A vector comprising the nucleic acid of Claim 48.
- 40 53. A vector comprising the nucleic acid of Claim 48 operably linked to an expression control sequence.
54. A host cell comprising the vector of claim 53
- 45

55. An isolated nucleic acid comprising the nucleotide sequence that encodes the amino acid sequence of residues 23 through 502 of SEQ ID NO:12
56. An isolated nucleic acid having the nucleotide sequence that encodes the amino acid sequence of SEQ ID NO:12
57. The isolated nucleic acid of claim 55 which has the nucleotide sequence between positions 67 through 1509 of SEQ ID NO:11
58. The isolated nucleic acid of claim 56 having the nucleotide sequence of SEQ ID NO:11
59. A vector comprising the nucleic acid of Claim 55.
60. A vector comprising the nucleic acid of Claim 55 operably linked to an expression control sequence.
61. A host cell comprising the vector of claim 60
62. An isolated nucleic acid comprising the nucleotide sequence that encodes the amino acid sequence of residues 23 through 502 of SEQ ID NO:14
63. An isolated nucleic acid having the nucleotide sequence that encodes the amino acid sequence of SEQ ID NO:14
64. The isolated nucleic acid of claim 62 which has the nucleotide sequence between positions 67 through 1509 of SEQ ID NO:13
65. The isolated nucleic acid of claim 63 having the nucleotide sequence of SEQ ID NO:13
66. A vector comprising the nucleic acid of Claim 62.
67. A vector comprising the nucleic acid of Claim 62 operably linked to an expression control sequence.
68. A host cell comprising the vector of claim 67
69. An isolated nucleic acid comprising the nucleotides encoding amino acids 230 to 241 of SEQ ID NO: 6 wherein the codon that encodes the amino acid at position 230 encodes a proline and conservative substitutions of proline.
70. The isolated nucleic acid of claim 69 wherein the codon that encodes the amino acid at position 230 of SEQ ID NO: 6 encodes an amino acid selected from the group consisting of glycine, alanine, proline, isoleucine, leucine, and valine.

71. The isolated nucleic acid of claim 70 wherein the codon that encodes the amino acid at position 230 of SEQ ID NO: 6 encodes a proline.
72. A vector comprising the nucleic acid of Claim 69
- 5 73. A vector comprising the nucleic acid of Claim 69 operably linked to an expression control sequence.
74. A host cell comprising the vector of claim 73
- 10 75. An isolated nucleic acid comprising the nucleotides encoding amino acids 230 to 241 of SEQ ID NO: 6 wherein the codon that encodes the amino acid at position 241 encodes a serine and conservative substitutions of serine.
76. The isolated nucleic acid of claim 74 wherein the codon that encodes the amino acid at position 241 of SEQ ID NO: 6 encodes an amino acid selected from the group consisting of serine, threonine, methionine, asparagine, glutamine and tyrosine,
- 15 77. The isolated nucleic acid of claim 76 wherein the codon that encodes the amino acid at position 241 of SEQ ID NO: 6 encodes a serine.
- 20 78. A vector comprising the nucleic acid of Claim 75
79. A vector comprising the nucleic acid of Claim 75 operably linked to an expression control sequence.
- 25 80. A host cell comprising the vector of claim 79
81. A fluorescent ligand binding assay comprising:
incubating cells with a fluorescent ligand capable of binding to cell surface receptors; and
30 measuring the fluorescence of cell bound fluorescent ligand using FLIPR.
82. The fluorescent ligand binding assay of claim 81 comprising the additional step of washing away unbound fluorescent ligand prior to said measuring step.
- 35 83. The fluorescent ligand binding assay of Claim 81 wherein the cells are the host cells of Claims 47, 54, 61, 68, 74, or 80.
84. A method of screening compounds for $\alpha 7$ nAChR agonist activity comprising:

Incubating the host cells of Claims 47, 54, 61, 68, 74, or 80 with a test compound: and measuring channel activity.

85. The method of claim 84 wherein said channel activity is measured by accessing
5 calcium flux.

86. The method of claim 85 wherein the calcium flux is accessed by measuring ionic flux directly

87. The method of claim 85 wherein the calcium flux is measured using fluorescent
10 indicators

88. The method of claim 87 wherein the calcium flux is measured using FLIPR.

89. A method of screening compounds for $\alpha 7$ nAChR modulation activity comprising:

15 incubating the host cells of Claims 47, 54, 61, 68, 74, or 80 in the presence or the absence of a test compound followed by;
incubating with an $\alpha 7$ nAChR agonist and
comparing the channel activity in the presence and absence of said test compound.

20 90. The method of claim 89 wherein said test compound decreases the channel activity

91. The method of claim 89 wherein said test compound increases the channel activity

25 92. The method of claim 89 wherein said channel activity is measured by accessing calcium flux.

93. The method of claim 92 wherein the calcium flux is accessed by measuring ionic flux directly

30 94. The method of claim 93 wherein the calcium flux is measured using fluorescent indicators

95. The method of claim 94 wherein the calcium flux is measured using FLIPR.

96. The method of claim 89 wherein said $\alpha 7$ nAChR agonist is nicotine.
97. An isolated polypeptide comprising the amino acid sequence set forth in SEQ ID NO:6
- 5 98. An isolated polypeptide comprising residues 23 through 470 of SEQ ID NO:6
99. An isolated polypeptide comprising the amino acid sequence set forth in SEQ ID NO:6
100. An isolated polypeptide comprising the amino acid sequence set forth in SEQ ID NO:10
- 10 101. An isolated polypeptide comprising residues 23 through 502 of SEQ ID NO:10
102. An isolated polypeptide comprising the amino acid sequence set forth in SEQ ID NO:12
- 15 103. An isolated polypeptide comprising residues 23 through 502 of SEQ ID NO:12
104. An isolated polypeptide comprising the amino acid sequence set forth in SEQ ID NO:14
105. An isolated polypeptide comprising residues 23 through 502 of SEQ ID NO:14
- 20 106. An isolated polypeptide comprising the amino acids at position 230 through 241 of SEQ ID NO: 6 wherein the amino acid at position 230 is a proline or conservative substitutions of proline.
107. The isolated polypeptide of Claim 106 wherein the amino acid at position 230 of SEQ ID NO: 6 is an amino acid selected from the group consisting of glycine, alanine, proline, isoleucine, leucine, and valine.
- 25 108. The isolated polypeptide of claim 107 wherein the amino acid at position 230 of SEQ ID NO: 6 is a proline.
- 30 109. An isolated polypeptide comprising the amino acids at position 230 through 241 of SEQ ID NO: 6 wherein the amino acid at position 241 is a serine or conservative substitutions of serine

110. The isolated polypeptide of Claim 109 wherein the amino acid at position 241 of SEQ ID NO: 6 is an amino acid selected from the group consisting of serine, threonine, methionine, asparagine, glutamine and tyrosine.

- 5 111. The isolated polypeptide of claim 110 wherein the amino acid at position 241 of SEQ ID NO: 6 is a serine.

Figure 1

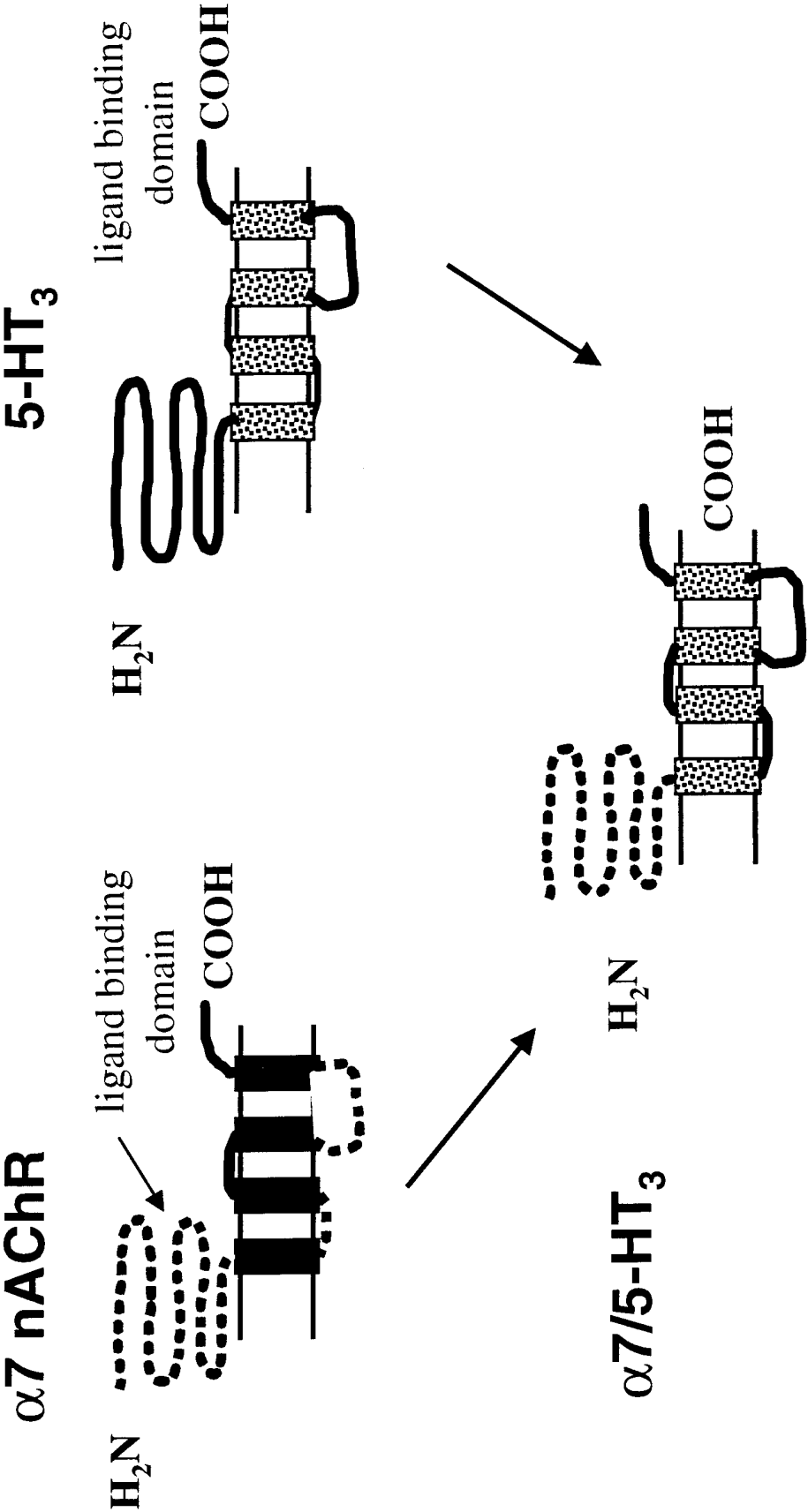


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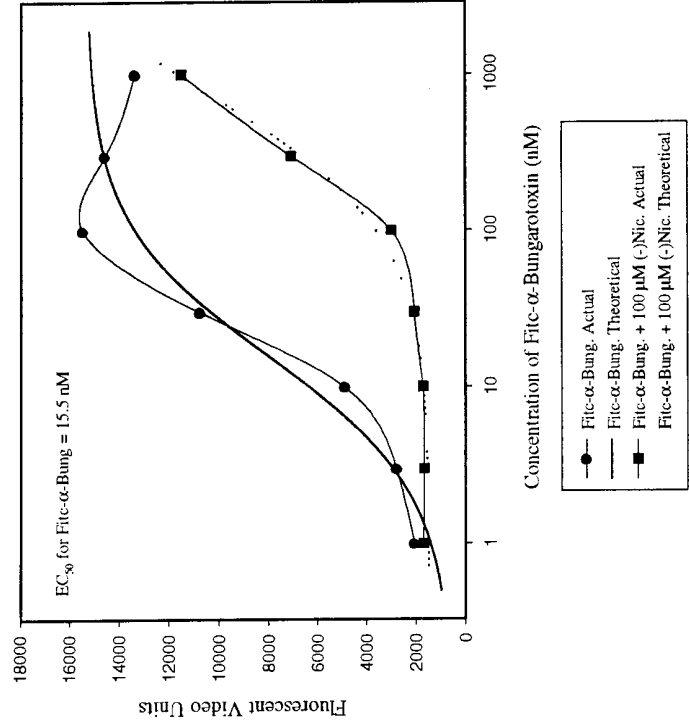


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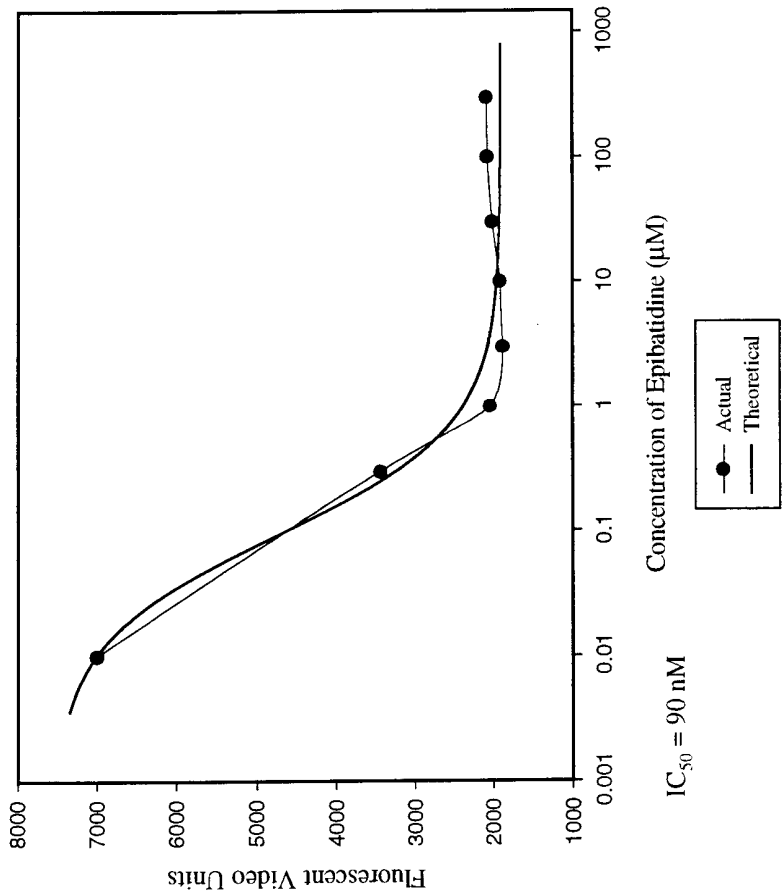


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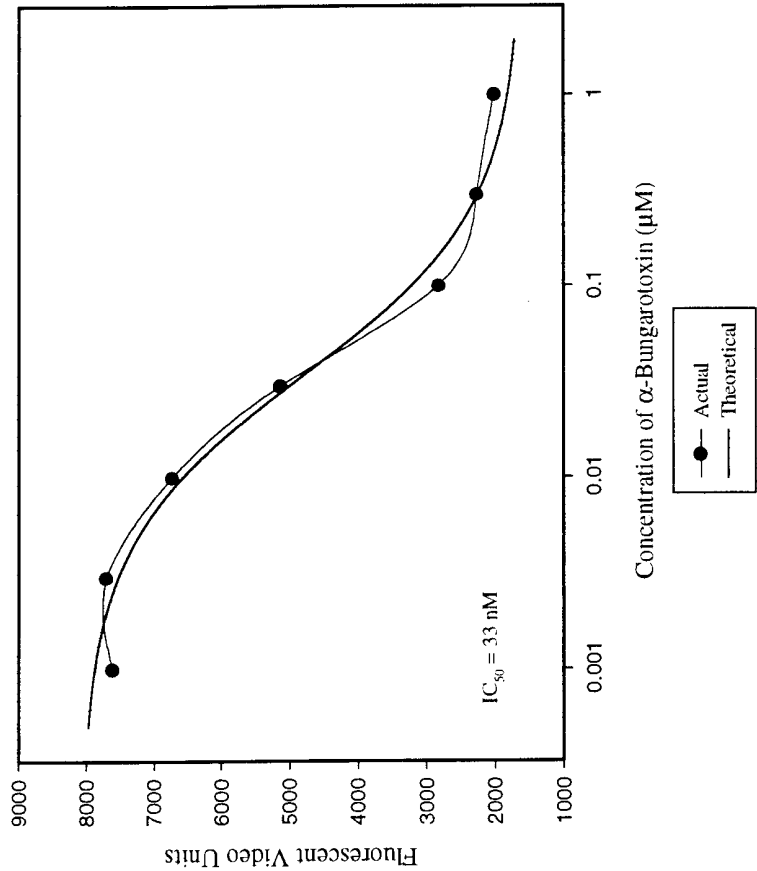


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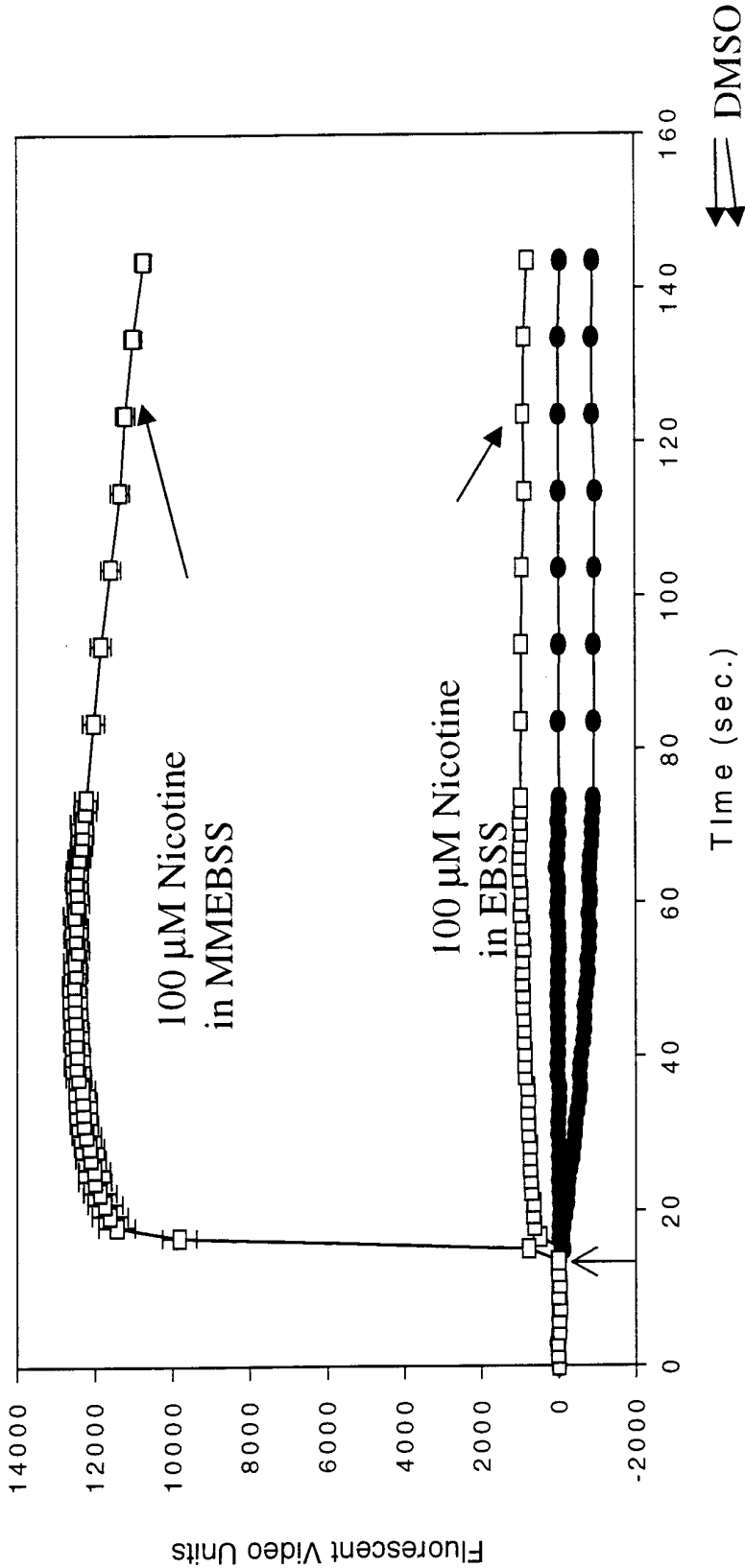


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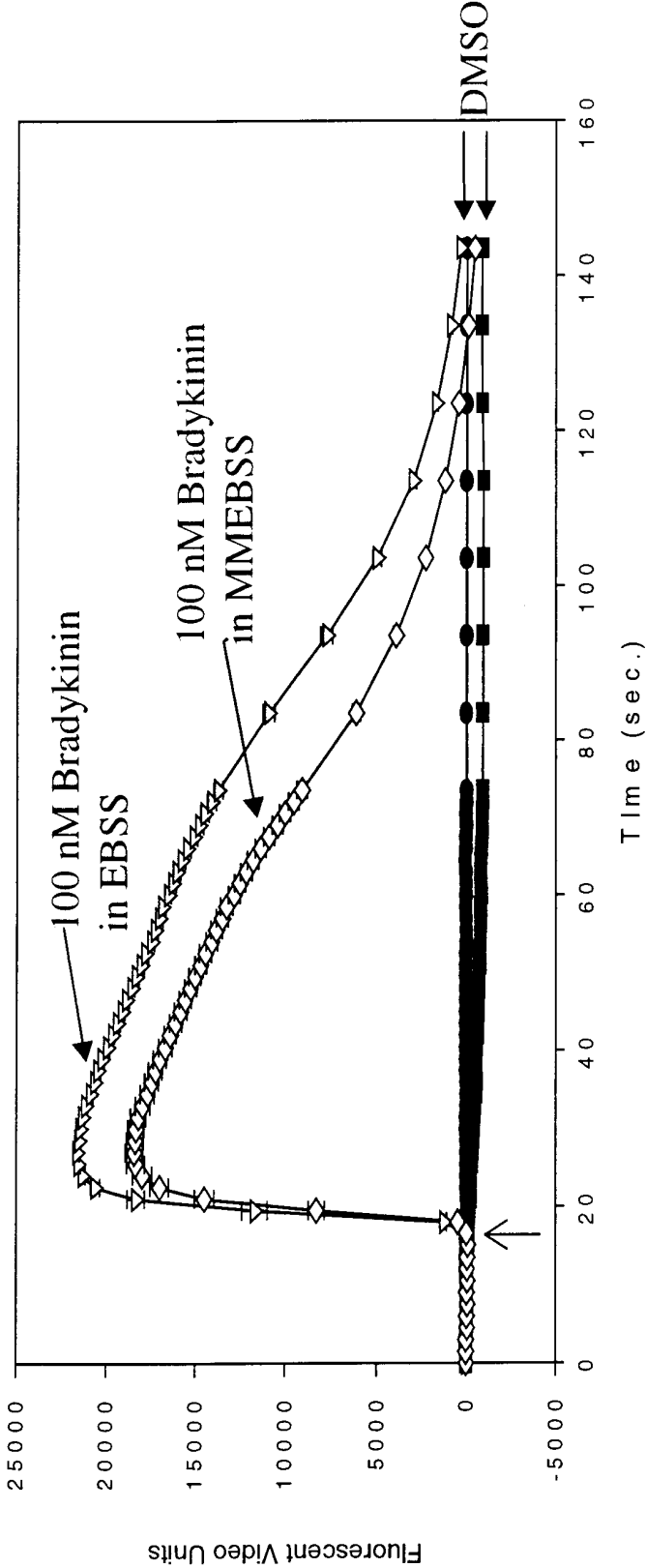


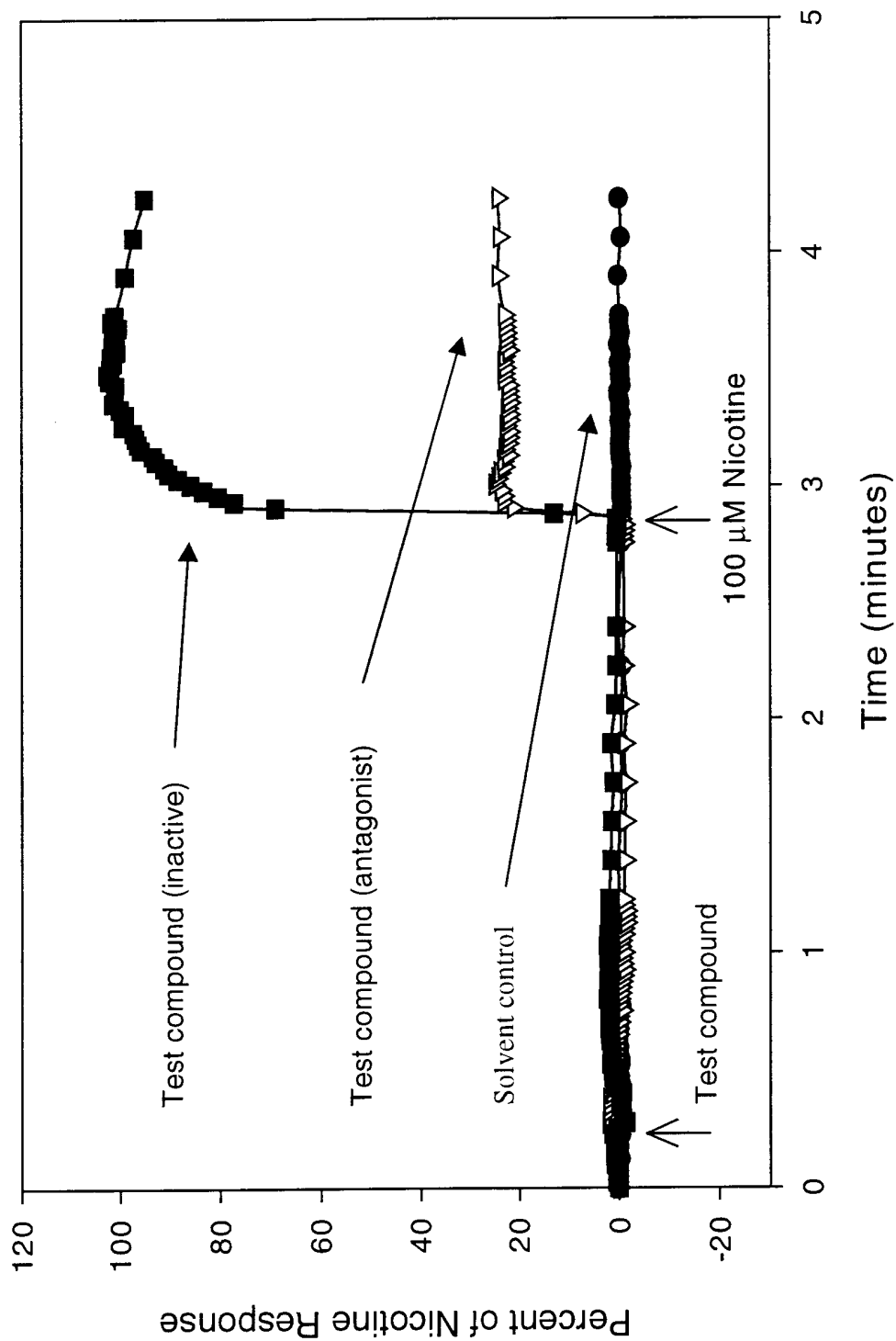
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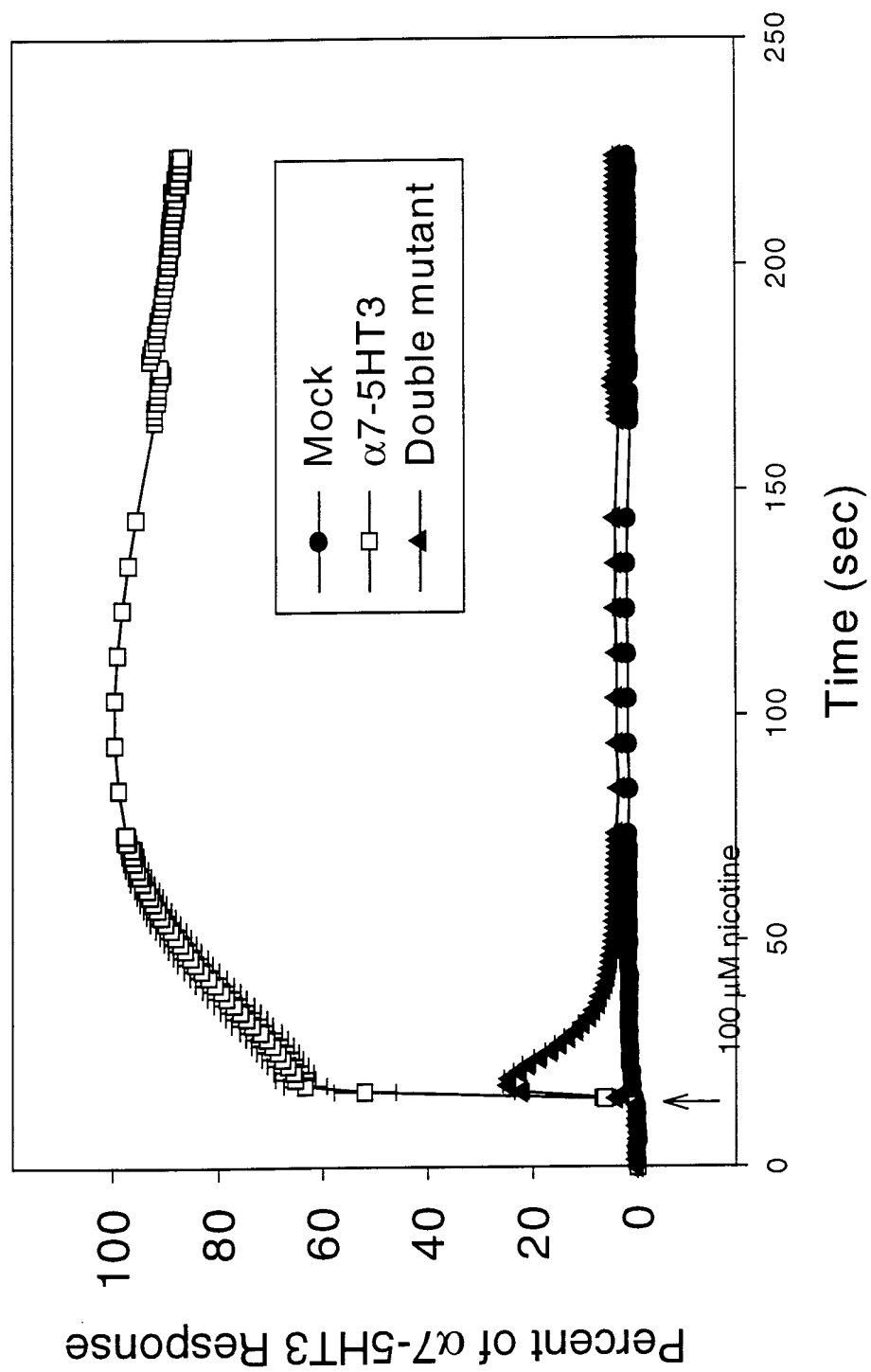
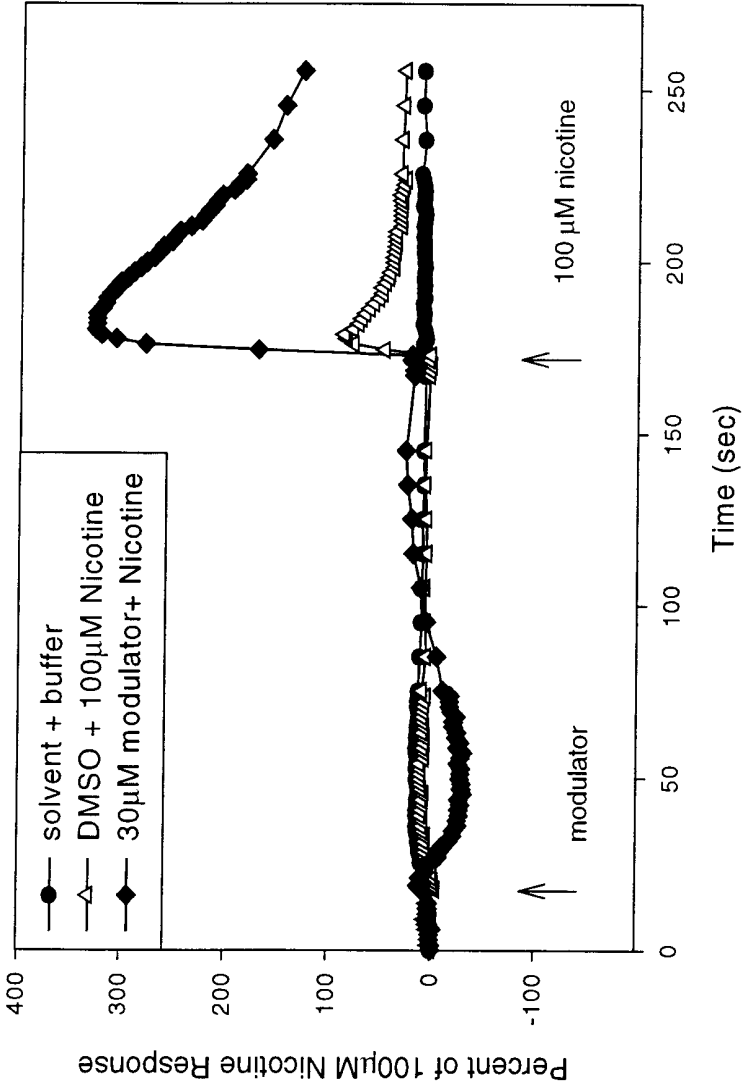
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Figure 10



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Wolfe, Mark L.

Berkenpas, Mitchell B

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Conductance

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<210> 10

<211> 502

<212> PRT

<213> Homo sapiens

<400> 10

Met Arg Cys Ser Pro Gly Gly Val Trp Leu Ala Leu Ala Ala Ser Leu
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Leu Val Lys Asn Tyr Asn Pro Leu Glu Arg Pro Val Ala Asn Asp Ser
 35 40 45

Gln Pro Leu Thr Val Tyr Phe Ser Leu Ser Leu Leu Gln Ile Met Asp
 50 55 60

Val Asp Glu Lys Asn Gln Val Leu Thr Thr Asn Ile Trp Leu Gln Met
 65 70 75 80

Ser Trp Thr Asp His Tyr Leu Gln Trp Asn Val Ser Glu Tyr Pro Gly
 85 90 95

Val Lys Thr Val Arg Phe Pro Asp Gly Gln Ile Trp Lys Pro Asp Ile
 100 105 110

Leu Leu Tyr Asn Ser Ala Asp Glu Arg Phe Asp Ala Thr Phe His Thr
 115 120 125

Asn Val Leu Val Asn Ser Ser Gly His Cys Gln Tyr Leu Pro Pro Gly
 130 135 140

Ile Phe Lys Ser Ser Cys Tyr Ile Asp Val Arg Trp Phe Pro Phe Asp
 145 150 155 160

Val Gln His Cys Lys Leu Lys Phe Gly Ser Trp Ser Tyr Gly Gly Trp
 165 170 175

Ser Leu Asp Leu Gln Met Gln Glu Ala Asp Ile Ser Gly Tyr Ile Pro
 180 185 190

Asn Gly Glu Trp Asp Leu Val Gly Ile Pro Gly Lys Arg Ser Glu Arg
 195 200 205

Phe Tyr Glu Cys Cys Lys Glu Pro Tyr Pro Asp Val Thr Phe Thr Val
 210 215 220

Thr Met Arg Arg Arg Pro Leu Tyr Tyr Gly Leu Asn Leu Leu Ile Pro

| | | | | | | |
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| Cys Val Leu Ile Ser Ala Leu Ala Leu Leu Val Phe Leu Leu Pro Ala | | | | | | |
| | 245 | | 250 | | 255 | |
| Asp Ser Gly Glu Lys Ile Ser Leu Gly Ile Thr Val Leu Leu Ser Leu | | | | | | |
| | 260 | | 265 | | 270 | |
| Thr Val Phe Met Leu Leu Val Ala Glu Ile Met Pro Ala Thr Ser Asp | | | | | | |
| | 275 | | 280 | | 285 | |
| Ser Val Pro Leu Ile Ala Gln Tyr Phe Ala Ser Thr Met Ile Ile Val | | | | | | |
| | 290 | | 295 | | 300 | |
| Gly Leu Ser Val Val Val Thr Val Ile Val Leu Gln Tyr His His His | | | | | | |
| 305 | | 310 | | 315 | | 320 |
| Asp Pro Asp Gly Gly Lys Met Pro Lys Trp Thr Arg Val Ile Leu Leu | | | | | | |
| | 325 | | 330 | | 335 | |
| Asn Trp Cys Ala Trp Phe Leu Arg Met Lys Arg Pro Gly Glu Asp Lys | | | | | | |
| | 340 | | 345 | | 350 | |
| Val Arg Pro Ala Cys Gln His Lys Gln Arg Arg Cys Ser Leu Ala Ser | | | | | | |
| | 355 | | 360 | | 365 | |
| Val Glu Met Ser Ala Val Ala Pro Pro Pro Ala Ser Asn Gly Asn Leu | | | | | | |
| | 370 | | 375 | | 380 | |
| Leu Tyr Ile Gly Phe Arg Gly Leu Asp Gly Val His Cys Val Pro Thr | | | | | | |
| 385 | | 390 | | 395 | | 400 |
| Pro Asp Ser Gly Val Val Cys Gly Arg Met Ala Cys Ser Pro Thr His | | | | | | |
| | 405 | | 410 | | 415 | |
| Asp Glu His Leu Leu His Gly Gly Gln Pro Pro Glu Gly Asp Pro Asp | | | | | | |
| | 420 | | 425 | | 430 | |
| Leu Ala Lys Ile Leu Glu Glu Val Arg Tyr Ile Ala Asn Arg Phe Arg | | | | | | |
| | 435 | | 440 | | 445 | |
| Cys Gln Asp Glu Ser Glu Ala Val Cys Ser Glu Trp Lys Phe Ala Ala | | | | | | |
| | 450 | | 455 | | 460 | |
| Cys Val Val Asp Arg Leu Cys Leu Met Ala Phe Ser Val Phe Thr Ile | | | | | | |
| 465 | | 470 | | 475 | | 480 |
| Ile Cys Thr Ile Gly Ile Leu Met Ser Ala Pro Asn Phe Val Glu Ala | | | | | | |

485

490

495

Val Ser Lys Asp Phe Ala
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<210> 11

<211> 1509

<212> DNA

<213> Homo sapiens

<400> 11

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cagatcatgg acgtggatga gaagaaccaa gttttaacca ccaacatttg gctgcaaata 240
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<210> 12

<211> 502

<212> PRT

<213> Homo sapiens

<400> 12

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Leu His Val Ser Leu Gln Gly Glu Phe Gln Arg Lys Leu Tyr Lys Glu
 20 25 30

Leu Val Lys Asn Tyr Asn Pro Leu Glu Arg Pro Val Ala Asn Asp Ser
 35 40 45

Gln Pro Leu Thr Val Tyr Phe Ser Leu Ser Leu Leu Gln Ile Met Asp
 50 55 60

Val Asp Glu Lys Asn Gln Val Leu Thr Thr Asn Ile Trp Leu Gln Met
 65 70 75 80

Ser Trp Thr Asp His Tyr Leu Gln Trp Asn Val Ser Glu Tyr Pro Gly
 85 90 95

Val Lys Thr Val Arg Phe Pro Asp Gly Gln Ile Trp Lys Pro Asp Ile
 100 105 110

Leu Leu Tyr Asn Ser Ala Asp Glu Arg Phe Asp Ala Thr Phe His Thr
 115 120 125

Asn Val Leu Val Asn Ser Ser Gly His Cys Gln Tyr Leu Pro Pro Gly
 130 135 140

Ile Phe Lys Ser Ser Cys Tyr Ile Asp Val Arg Trp Phe Pro Phe Asp
 145 150 155 160

Val Gln His Cys Lys Leu Lys Phe Gly Ser Trp Ser Tyr Gly Gly Trp
 165 170 175

Ser Leu Asp Leu Gln Met Gln Glu Ala Asp Ile Ser Gly Tyr Ile Pro
 180 185 190

Asn Gly Glu Trp Asp Leu Val Gly Ile Pro Gly Lys Arg Ser Glu Arg
 195 200 205

Phe Tyr Glu Cys Cys Lys Glu Pro Tyr Pro Asp Val Thr Phe Thr Val
 210 215 220

Thr Met Arg Arg Arg Thr Leu Tyr Tyr Gly Leu Asn Leu Leu Ile Pro
 225 230 235 240

Ser Val Leu Ile Ser Ala Leu Ala Leu Leu Val Phe Leu Leu Pro Ala
 245 250 255

Asp Ser Gly Glu Lys Ile Ser Leu Gly Ile Thr Val Leu Leu Ser Leu
 260 265 270

Thr Val Phe Met Leu Leu Val Ala Glu Ile Met Pro Ala Thr Ser Asp
 275 280 285

Ser Val Pro Leu Ile Ala Gln Tyr Phe Ala Ser Thr Met Ile Ile Val
 290 295 300

Gly Leu Ser Val Val Val Thr Val Ile Val Leu Gln Tyr His His His
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Asp Pro Asp Gly Gly Lys Met Pro Lys Trp Thr Arg Val Ile Leu Leu
 325 330 335

Asn Trp Cys Ala Trp Phe Leu Arg Met Lys Arg Pro Gly Glu Asp Lys
 340 345 350

Val Arg Pro Ala Cys Gln His Lys Gln Arg Arg Cys Ser Leu Ala Ser
 355 360 365

Val Glu Met Ser Ala Val Ala Pro Pro Pro Ala Ser Asn Gly Asn Leu
 370 375 380

Leu Tyr Ile Gly Phe Arg Gly Leu Asp Gly Val His Cys Val Pro Thr
 385 390 395 400

Pro Asp Ser Gly Val Val Cys Gly Arg Met Ala Cys Ser Pro Thr His
 405 410 415

Asp Glu His Leu Leu His Gly Gly Gln Pro Pro Glu Gly Asp Pro Asp
 420 425 430

Leu Ala Lys Ile Leu Glu Glu Val Arg Tyr Ile Ala Asn Arg Phe Arg
 435 440 445

Cys Gln Asp Glu Ser Glu Ala Val Cys Ser Glu Trp Lys Phe Ala Ala
 450 455 460

Cys Val Val Asp Arg Leu Cys Leu Met Ala Phe Ser Val Phe Thr Ile
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Ile Cys Thr Ile Gly Ile Leu Met Ser Ala Pro Asn Phe Val Glu Ala
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Val Ser Lys Asp Phe Ala
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<210> 13

<211> 1509

<212> DNA

<213> Homo sapiens

<400> 13

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<210> 14

<211> 502

<212> PRT

<213> Homo sapiens

<400> 14

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             20             25             30

Leu Val Lys Asn Tyr Asn Pro Leu Glu Arg Pro Val Ala Asn Asp Ser
  35             40             45

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Gln Pro Leu Thr Val Tyr Phe Ser Leu Ser Leu Leu Gln Ile Met Asp
 50 55 60

Val Asp Glu Lys Asn Gln Val Leu Thr Thr Asn Ile Trp Leu Gln Met
 65 70 75 80

Ser Trp Thr Asp His Tyr Leu Gln Trp Asn Val Ser Glu Tyr Pro Gly
 85 90 95

Val Lys Thr Val Arg Phe Pro Asp Gly Gln Ile Trp Lys Pro Asp Ile
 100 105 110

Leu Leu Tyr Asn Ser Ala Asp Glu Arg Phe Asp Ala Thr Phe His Thr
 115 120 125

Asn Val Leu Val Asn Ser Ser Gly His Cys Gln Tyr Leu Pro Pro Gly
 130 135 140

Ile Phe Lys Ser Ser Cys Tyr Ile Asp Val Arg Trp Phe Pro Phe Asp
 145 150 155 160

Val Gln His Cys Lys Leu Lys Phe Gly Ser Trp Ser Tyr Gly Gly Trp
 165 170 175

Ser Leu Asp Leu Gln Met Gln Glu Ala Asp Ile Ser Gly Tyr Ile Pro
 180 185 190

Asn Gly Glu Trp Asp Leu Val Gly Ile Pro Gly Lys Arg Ser Glu Arg
 195 200 205

Phe Tyr Glu Cys Cys Lys Glu Pro Tyr Pro Asp Val Thr Phe Thr Val
 210 215 220

Thr Met Arg Arg Arg Pro Leu Tyr Tyr Gly Leu Asn Leu Leu Ile Pro
 225 230 235 240

Ser Val Leu Ile Ser Ala Leu Ala Leu Leu Val Phe Leu Leu Pro Ala
 245 250 255

Asp Ser Gly Glu Lys Ile Ser Leu Gly Ile Thr Val Leu Leu Ser Leu
 260 265 270

Thr Val Phe Met Leu Leu Val Ala Glu Ile Met Pro Ala Thr Ser Asp
 275 280 285

Ser Val Pro Leu Ile Ala Gln Tyr Phe Ala Ser Thr Met Ile Ile Val
 290 295 300

Gly Leu Ser Val Val Val Thr Val Ile Val Leu Gln Tyr His His His
 305 310 315 320
 Asp Pro Asp Gly Gly Lys Met Pro Lys Trp Thr Arg Val Ile Leu Leu
 325 330 335
 Asn Trp Cys Ala Trp Phe Leu Arg Met Lys Arg Pro Gly Glu Asp Lys
 340 345 350
 Val Arg Pro Ala Cys Gln His Lys Gln Arg Arg Cys Ser Leu Ala Ser
 355 360 365
 Val Glu Met Ser Ala Val Ala Pro Pro Pro Ala Ser Asn Gly Asn Leu
 370 375 380
 Leu Tyr Ile Gly Phe Arg Gly Leu Asp Gly Val His Cys Val Pro Thr
 385 390 395 400
 Pro Asp Ser Gly Val Val Cys Gly Arg Met Ala Cys Ser Pro Thr His
 405 410 415
 Asp Glu His Leu Leu His Gly Gly Gln Pro Pro Glu Gly Asp Pro Asp
 420 425 430
 Leu Ala Lys Ile Leu Glu Glu Val Arg Tyr Ile Ala Asn Arg Phe Arg
 435 440 445
 Cys Gln Asp Glu Ser Glu Ala Val Cys Ser Glu Trp Lys Phe Ala Ala
 450 455 460
 Cys Val Val Asp Arg Leu Cys Leu Met Ala Phe Ser Val Phe Thr Ile
 465 470 475 480
 Ile Cys Thr Ile Gly Ile Leu Met Ser Ala Pro Asn Phe Val Glu Ala
 485 490 495
 Val Ser Lys Asp Phe Ala
 500