



(51) International Patent Classification:
G01N 33/566 (2006.01) *C12N 15/10* (2006.01)
C07K 14/72 (2006.01)

(21) International Application Number:
PCT/DK2010/050094

(22) International Filing Date:
26 April 2010 (26.04.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/174,203 30 April 2009 (30.04.2009) US

(71) Applicant (for all designated States except US): **H. LUNDBECK A/S** [DK/DK]; Ottiliavej 9, DK-2500 Valby (DK).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **THIRSTRUP, Kenneth** [DK/DK]; Søborg Parkallé 40, DK-2860 Søborg (DK).

(74) Common Representative: **H. LUNDBECK A/S**; Corporate Patents, Ottiliavej 9, DK-2500 Valby (DK).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM,

[Continued on next page]

(54) Title: CONSTITUTIVELY ACTIVE MUTANTS AND USES THEREOF

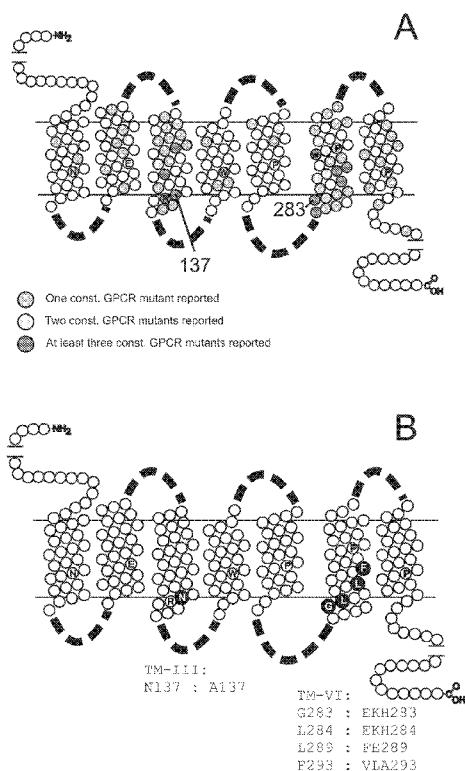


Figure 1.

(57) Abstract: The present invention provides a method for developing an assay suitable for screening of compounds to modulate the activity of a receptor, particularly a constitutively active receptor. Another aspect of the invention provides a constitutively active G protein-coupled receptor, namely GPR88. Another aspect of the invention is a mutated constitutively active GPR88, i.e., having increased basal activity relative to a wild-type GPR88 polypeptide, and a modified reporter gene which provides for a faster assay readout.



TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

Published:

— upon request of the applicant, before the expiration of the time limit referred to in Article 21(2)(a)

— with sequence listing part of description (Rule 5.2(a))

CONSTITUTIVELY ACTIVE MUTANTS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a PCT International Application claiming the benefit of priority
5 of U.S. Provisional Application No. 61/174,203, filed April 30, 2009, which is hereby
incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The present application contains a Sequence Listing, submitted in Computer Readable
Form containing Filename 0661-WO-PCT_ST25_SeqListing.txt, of size 72,800 bytes, created on
10 April 21, 2010. The sequence listing is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention provides methods for creating and assaying non-endogenous
constitutively active G protein-coupled receptors. The present invention further provides
methods for identifying compounds that bind to, activate or inhibit activation of constitutively
15 active G protein-coupled receptors. The present invention also provides an optimized assay for
detecting or measuring G protein-coupled receptor activity.

BACKGROUND OF THE INVENTION

[0004] Throughout this application various publications are referred to by citations within
parenthesis. The disclosures of these publications, in their entireties, are hereby incorporated by
20 reference into this application in order to more fully describe the state of the art to which the
invention pertains.

[0005] G protein-coupled receptors (GPCRs) having altered signaling are important tools for
drug discovery due to the fact that a considerable number of diseases and other adverse effects
can result from abnormal receptor activity. Similarly, it is important to identify mutant or
25 polymorphic receptors where the mutation or polymorphism alters the response of the receptor to
a particular ligand, for example, a drug or peptide hormone. Receptors having altered signaling

include receptors that display a change in ligand dependent or independent (basal) signaling. For example, ligand-dependent receptors might display an increase or decrease in signaling. Ligand-dependent receptors that have an increased sensitivity to ligand stimulation include hypersensitive receptors and receptors having increased potency.

5 [0006] A major focus of research is the identification of receptors and ligands which affect cellular processes which correlate to various diseases and conditions. Many diseases or conditions are thought to be caused by improper intracellular signaling, in which biological molecules are produced in excessive or insufficient quantities. Many of these biological molecules may be produced by activated receptors even in the absence of a ligand, i.e., receptors
10 which are constitutively active. Thus, identification of receptors which are constitutively active is particularly desirable. For example, compounds may be screened against a constitutively active receptor in order to determine whether any compounds may alter the receptor activity, and would thus represent potential drugs for the treatment of diseases or conditions caused by the increased receptor activity.

15 [0007] Methods of identifying receptors having altered signaling that can be used in high throughput drug screening assays have not been straight-forward. For example, it has been particularly challenging to identify receptors having alterations in basal signaling, for example, constitutively active receptors. Constitutively active receptors are particularly valuable as sensitive detection systems for drug discovery. In general, a number of publications describe
20 different mutations in G protein-coupled receptors which results in enhanced basal activity of the receptor, and Figure 1 is provided as a summary of such reported mutations.

[0008] G protein-coupled receptors constitute a superfamily of diverse proteins with hundreds of members, and act as receptors for a multitude of different signals and are targets for pharmaceutical products. Several categories of GPCRs are selectively expressed in subsets of
25 cells and tissues. For example, chemosensory GPCRs are receptors for sensory signals of external origin that are sensed as odors, pheromones, or tastes. Most other G protein-coupled receptors respond to endogenous signals, or ligands, such as peptides, lipids, neurotransmitters, or nucleotides. G protein-coupled receptors falling in the latter group are involved in numerous physiological processes, including the regulation of neuronal excitability, metabolism,

reproduction, development, hormonal homeostasis, and behavior, and are differentially expressed in many cell types in the body.

[0009] A large number of G protein-coupled receptors are found in the brain. Excluding the large family of odorant receptors, over 89% of known G protein coupled-receptors are active in the brain. It has been hypothesized that many of these receptors serve as modulators of behavior, memory, cognition, pain, and instinctive functions. G protein coupled-receptors especially those in the nervous system, are ideal targets for drug development. The preference for GPCRs as specific drug targets derives, not only from their central role in biological processes, but also from the discriminating ability that these molecules have in recognizing and responding to their signals. Many G protein coupled-receptors exist in several similar, but subtly distinct subtypes, which are found in different cells in the body. Such variety of sequence and location provides a high degree of selectivity, allowing the discovery of drugs which specifically affect one subtype of receptor, but not another. This selectivity substantially reduces the risk of unwanted side effects. In addition, most G protein coupled-receptors are located in the plasma membranes of cells, where they can be easily accessed by pharmaceutical compounds. Given these properties, G protein coupled-receptors, as a group, have emerged among the most coveted targets for drug development. Thus, there exists a need to identify GPCRs associated with specific diseases, including neurological or metabolic diseases and disorders.

[0010] Identification of endogenous and non-endogenous constitutively active receptors have proved particularly valuable as sensitive detection systems for use in drug discovery. Furthermore, constitutively active receptors are utilized in distinguishing inverse agonists from neutral antagonists, which modulators may have differential effects in certain receptor systems. For example, others have shown that inverse agonists may be a preferred pharmaceutical choice over neutral antagonists in modulating the histamine H3 receptor (Morisset, S. et al., 2000, *Nature*, 374: 272-276). Also compounds determined to be inverse agonists of the constitutively active form of the human β_2 -adrenergic receptor caused an upregulation of receptor sites, whereas neutral antagonists did not have this effect (MacEwan, J. and Milligan, G., 1996, *FEBS Letters*, 399:108-112). Constitutively active receptors are therefore useful in identifying compounds with enhanced sensitivity and beneficial biological effects.

[0011] Constitutively active proteins have also been utilized in cell transplantation. Constitutively active AKT (protein kinase B) enhances the activity and/or proliferation of human islet transplants in a mouse model of diabetes, thereby reducing the number of cells sequestered and transplanted as well as improving the therapeutic outcome (Rao, P. et al., 2005, *Diabetes*, 54:1664-1674).

[0012] GPR88 is a G protein-coupled receptor highly expressed in the striatum (Mizushima, K. et al., 2000, *Genomics*, 69:314-312). As provided by WO 2008/061209, GPR88 knock-out mice have reduced motor coordination, are hyperactive in the open field, are hypersensitive to amphetamine induced activity, and are sensitive to amphetamine-induced reduction of prepulse inhibition. GPR88 is suspected in playing a role in neurological diseases, such as Parkinson's Disease, Huntington's Disease, restless leg syndrome or other movement disorders, schizophrenia, drug addiction, and epilepsy.

SUMMARY OF THE INVENTION

[0013] An aspect of the invention provides a method for assaying a compound which modulates the activity of a G protein-coupled receptor, comprising: isolating and mutating a nucleic acid molecule encoding a wild-type G protein-coupled receptor, wherein mutating the nucleic acid molecule comprises creating at least one nucleotide mutation resulting in an amino acid change in the wild-type G protein-coupled receptor, such amino acid being expressed in transmembrane domain III or VI, thereby producing a second nucleic acid molecule sequence encoding a mutant receptor; separately assaying the basal activity of the wild-type receptor, the mutant receptor and a negative control; determining whether the activity of the mutant receptor is greater than the wild-type receptor and a negative control; and if the basal activity of the mutant receptor is greater, then assaying the compound at the mutant receptor to determine whether the compound is an agonist, inverse agonist or antagonist of the G protein-coupled receptor; and wherein the G protein-coupled receptor is human GPR88, or a variant thereof.

[0014] Another aspect of the invention provides for a method for detecting or measuring G protein-coupled receptor activity comprising: cotransfecting a host cell with an expression vector comprising a promoter operably linked to a nucleic acid molecule encoding a constitutively active mutant G protein-coupled receptor, wherein the receptor can induce a ligand-independent intracellular signal, and a reporter construct comprising a response element operably linked to a reporter gene, wherein the reporter gene is a modified reporter gene that is rapidly expressed by the intracellular signal induced by the receptor, and detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof within 24 hours of incubation time following the cotransfection of the expression vector and reporter construct.

[0015] Another aspect of the invention provides a modified reporter gene comprising SEQ ID NO: 28.

[0016] Another aspect of the invention provides a constitutively active mutant G protein-coupled receptor comprising a polypeptide encoded by SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26, or variants thereof.

[0017] Another aspect of the invention provides a constitutively active mutant G protein-coupled receptor comprising a polypeptide having the amino acid sequence of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 or 27, or variants thereof.

[0018] One aspect of the invention provides an isolated nucleic acid molecule comprising SEQ ID NO: 3 which encodes for a human GPR88 receptor with unaltered receptor activity.

[0019] Another aspect of the invention provides an isolated nucleic acid molecule comprising SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26, or variants thereof; or vectors comprising any of the isolated nucleic acid molecules of the invention; or cells transfected with any of the isolated nucleic acid molecules or the vectors of the invention.

[0020] Another aspect of the invention provides an isolated nucleic acid molecule which is complementary to SEQ ID NO:3.

[0021] Another aspect of the invention provides an isolated nucleic acid molecule encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, or variants thereof.

[0022] Another aspect of the invention provides an isolated polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, or variants thereof.

[0023] A further aspect of the invention provides a fragment of the polypeptides of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, wherein the fragment comprises an amino acid sequence having at least 293 contiguous amino acids. A further aspect of the invention provides a fragment of the polypeptides of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 or 27,

wherein the fragment comprises an amino acid sequence having at least 50 contiguous amino acids.

[0024] A further aspect of the invention provides a fragment of SEQ ID NO: 5 comprising histidine (H) at amino acid position 283. An aspect of the invention provides a fragment of SEQ ID NO: 7 comprising lysine (K) at amino acid position 284. An aspect of the invention provides a fragment of SEQ ID NO: 9 comprising glutamic acid (E) at amino acid position 284. An aspect of the invention provides a fragment of SEQ ID NO: 11 comprising glutamic acid (E) at amino acid position 283. An aspect of the invention provides a fragment of SEQ ID NO: 13 comprising lysine (K) at amino acid position 283. An aspect of the invention provides a fragment of SEQ ID NO: 15 comprising histidine (H) at amino acid position 284. An aspect of the invention provides a fragment of SEQ ID NO: 17 comprising alanine (A) at amino acid position 293. An aspect of the invention provides a fragment of SEQ ID NO: 19 comprising leucine (L) at amino acid position 293. An aspect of the invention provides a fragment of SEQ ID NO: 21 comprising valine (V) at amino acid position 293. An aspect of the invention provides a fragment of SEQ ID NO: 23 comprising glutamic acid (E) at amino acid position 289. An aspect of the invention provides a fragment of SEQ ID NO: 25 comprising phenylalanine (F) at amino acid position 289. An aspect of the invention provides a fragment of SEQ ID NO: 21 comprising valine (V) at amino acid position 293. A further aspect of the invention provides a fragment of SEQ ID NO: 27 comprising alanine (A) at amino acid position 137.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figure 1 provides a general overview of mutated G-protein coupled receptors that demonstrate constitutive activity. (A) Positions are indicated which correspond to relevant amino acid position numbers in the GPR88 receptor protein. (B) Amino acids positioned in transmembrane (TM) domains III and VI were mutated as detailed in Example 2.

[0026] Figure 2 provides a linear overview of the amino acid sequence of the GPR88 polypeptide (SEQ ID NO: 2), indicating the relevant amino acid positions of transmembrane domains I through VII (TMI-TMVII) by underlining, and the relevant amino acid positions selected for mutation within TMIII and TMVI are in bold.

[0027] Figure 3 provides an overview of the general principle behind the assay described in Examples 3 and 4.

[0028] Figure 4 shows results of the relative activity of GPR88 receptor mutants and appropriate controls, as detailed in Example 3.

[0029] Figure 5 shows a dose response activity relationship of increasing the amount of transfected G283H GPR88 DNA (SEQ ID NO: 4) in cells compared to wild-type hGPR88 (SEQ ID NO:1) as detailed in Example 3.

[0030] Figure 6 illustrates increased window and reduced variability in the GPR88 constitutively active receptor mutant assay as detailed in Example 3.

[0031] Figure 7 provides an illustration of an optimized luciferase construct KT-001, as detailed in Example 4, for use in the GPR88 constitutively active receptor mutant assay.

[0032] Figure 8 Illustrates the effect of using the optimized luciferase construct KT-001 in the GPR88 constitutively active receptor mutant assay, as detailed in Example 4. The results indicate that the optimized construct provides a shortened incubation time (Fig. 8B) and therefore a more rapid readout of the assay, as compared to a typical luciferase construct used in the assay (Fig. 8A).

[0033] DETAILED DESCRIPTION

[0034] The following amino acid abbreviations are used herein (IUPAC Joint Commission of Biochemical Nomenclature, "Nomenclature and Symbolism for Amino Acids and Peptides", 1984, *Eur. J. Biochem.* 138:9-37):

5

[0035] <u>Abbreviation</u>		<u>Amino acid name</u>
	Ala A	Alanine
	Arg R	Arginine
	Asn N	Asparagine
10	Asp D	Aspartic acid (Aspartate)
	Cys C	Cysteine
	Gln Q	Glutamine
	Glu E	Glutamic acid (Glutamate)
	Gly G	Glycine
15	His H	Histidine
	Ile I	Isoleucine
	Leu L	Leucine
	Lys K	Lysine
	Met M	Methionine
20	Phe F	Phenylalanine
	Pro P	Proline
	Ser S	Serine
	Thr T	Threonine
	Trp W	Tryptophan
25	Tyr Y	Tyrosine
	Val V	Valine
	Asx B	Aspartic acid or Asparagine
	Glx Z	Glutamine or Glutamic acid.
	Xaa X	Any amino acid.
30	TERM	termination codon

[0036] A "non-endogenous constitutively active receptor" is a receptor with a higher basal activity level than the corresponding wild-type receptor or a receptor possessing the ability to spontaneously signal in the absence of activation by a positive agonist. This term includes a constitutively active mutant receptor. This term also includes wild-type GPR88 receptors of any orthologues origin (from any species) that are naturally constitutively active (e.g., naturally occurring receptors, including naturally occurring polymorphic receptors and wild-type

35

receptors) and that have a higher basal activity level than a corresponding vector lacking a gene encoding a receptor. The constitutive activity of a receptor may also be established by comparing the basal level of signaling, such as second messenger signaling, of a mutant receptor to the basal level of signaling of the wild-type receptor. A constitutively active receptor exhibits at least a
5 25%, 50%, 75%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, or 900% increase in basal activity, and preferably more than a 1000% increase in basal level activity, compared to either the negative control or the wild-type receptor. The basal activity of a constitutively active receptor can be confirmed by its decrease in the presence of an inverse agonist.

[0037] "Basal" activity means the level of activity (e.g., activation of a specific biochemical
10 pathway or second messenger signaling event) of a receptor in the absence of stimulation with a receptor-specific ligand (e.g., a positive agonist). Preferably, basal activity is less than the level of ligand-stimulated activity of an appropriate control or wild-type receptor. However, in certain cases, a mutant receptor with increased basal activity might display a level of signaling that approximates, is equal to, or even exceeds the level of ligand-stimulated activity of the
15 corresponding wild-type receptor or appropriate control.

[0038] A "naturally-occurring" receptor refers to a form or sequence of a receptor as it exists in an animal, or to a form of the receptor that is homologous to the sequence known to those skilled in the art as the "wild-type" sequence. Those skilled in the art will understand "wild-type" receptor to refer to the conventionally accepted "wild-type" amino acid consensus sequence of
20 the receptor, or to a "naturally-occurring" receptor with normal physiological patterns of ligand binding and signaling.

[0039] A "mutant receptor" is a form of the receptor in which one or more amino acid residues in the predominant receptor occurring in nature, e.g., a naturally-occurring wild-type receptor, have been either deleted or replaced. Alternatively, additional amino acid residues may have been
25 inserted.

[0040] "Altered signaling" is a change in the ligand-dependent or ligand-independent signal typically generated by a receptor, as measured by the parameters of efficacy, potency, or basal signaling. The change or alteration may be an increase or decrease in ligand-dependent or ligand-

independent signaling. Examples of alterations in signaling include receptors having an increased sensitivity to ligand, i.e., hypersensitive receptors. This increased sensitivity to ligand may occur in the form of increased potency or increased efficacy in response to agonist stimulation. Other examples of receptors having alterations in signaling include receptors exhibiting a decreased sensitivity to ligand (i.e., hyposensitive or silenced receptors), receptors exhibiting a change in basal activity (e.g., receptors having an increased level of basal signaling, such as constitutively active receptors, or receptors having a decreased level of basal signaling, such as receptors having silencing mutations, i.e., fully silenced or partially silenced receptors). A receptor with altered signaling may exhibit a change in basal or ligand induced signaling or efficacy or potency relative to an appropriate negative control, e.g., that is considered statistically significant using accepted methods of statistical analysis.

[0041] A “substantially pure nucleic acid” is meant nucleic acid (e.g., DNA or RNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

[0042] “Transformed cell” means a cell into which (or into an ancestor of which) a DNA molecule encoding (as used herein) a polypeptide described herein has been introduced, by means of recombinant DNA techniques, such as transfection or transformation techniques, well-known to the person skilled in the art.

[0043] “Promoter” means a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific or tissue-specific regulators; or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene. A promoter element may be positioned for expression if it is positioned adjacent to a DNA sequence so it can direct transcription of the sequence.

[0044] "Operably linked" means that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

[0045] "Expression vectors" contain a promoter operably linked to the gene to be expressed.

5 [0046] A "reporter construct" contains a promoter operably linked to a reporter gene. Such reporter genes may be detected directly (e.g., by visual inspection or detection through an instrument) or indirectly (e.g., by binding of an antibody to the reporter gene product or by reporter product-mediated induction of a second gene product). Reporter genes are known in the art, and include genes encoding luciferase, green fluorescent protein, beta-galactosidase, secreted
10 alkaline phosphatase (SEAP), or chloramphenicol acetyl transferase (CAT) polypeptides (see, for example, Sambrook, J. et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, N.Y., or Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates, New York, N.Y., V 1-3,2000, incorporated herein by reference). Expression of the reporter gene may be detectable by use of an assay that directly or indirectly measures the
15 activity of the polypeptide encoded by the reporter gene. In one example, activity of the polypeptide encoded by the reporter gene is measured by detecting the polypeptide's enzymatic activity by reducing a substrate, and measuring the accumulation of the substrate byproducts. Preferred reporter constructs also include a response element.

[0047] A "response element" is a nucleic acid sequence that is sensitive to a particular signaling
20 pathway, e.g., a second messenger signaling pathway, and assists in driving transcription of the reporter gene. Response elements are well known in the art, and may be the promoter.

[0048] "Transactivating factors" are transcription factors that become activated through cellular signal transduction (usually induced by activation of a receptor), thereby translocating to the nucleus to activate transcription of a gene. Transactivating factors are well-known in the art.
25 Transactivating factors that may be used in the described methods include, but are not limited to, cAMP response element (CRE)-binding protein (CREB), Gal4, Elk1, NF- κ B, NF-IL6, NFAT, AP-1 and c-Jun. Transactivating factors that are encoded by the constructs of the invention may be fusion proteins containing the DNA binding domain of one transcription factor and the

transactivation domain of another transcription factor. Fusion transactivating factors are also well-known in the art.

[0049] A "second messenger signaling activity", also known as "second messenger response" refers to production of an intracellular stimulus (including, but not limited to, cAMP, cGMP, ppGpp, inositol phosphate, or calcium ions) in response to activation of the receptor, or to activation of a protein in response to receptor activation, including but not limited to a kinase, a phosphatase, or to activation or inhibition of a membrane channel.

[0050] A "negative control" is any construct that can be used to distinguish alterations in the signaling of a candidate receptor. The appropriate negative control for any given candidate receptor will vary depending on the assay and the type of alteration in signaling. For example, to identify a constitutively active receptor, the appropriate negative controls may be an empty vector negative control, i.e. a vector lacking any receptor nucleotide sequences, or a vector including nonconstitutively active wild-type receptor nucleotide sequences. The appropriate negative control to be used to identify a receptor with altered signaling will be apparent to a person of ordinary skill in the art.

[0051] The present invention provides constitutively active receptors, e.g., mutant receptors, and use of such receptors in the identification of compounds which may be useful in treating various diseases and conditions, e.g., which result from improper receptor signaling.

[0052] The mutated receptors, or mutant receptors, of the present invention can be used in assays to identify ligands (e.g., peptides, and small molecules) that bind to and activate, or inhibit activation of the receptor. Identifying ligands that are agonists or antagonists of a receptor is the first step in narrowing the pool of candidate ligands that can be targeted for further analysis in potential therapeutic treatments. The goal of such analysis is to identify and characterize useful ligands which may be useful in the treatment of a disease or condition. Indeed, receptors having altered signaling, such as the constitutively active mutant receptors of the present invention, are important tools for drug discovery due to the fact that a considerable number of diseases and other adverse effects can result from abnormal receptor activity. Thus, by using the

constitutively active receptors of the present invention and their corresponding wild-type receptors, ligands of the receptors may be identified that are key therapeutic agents.

[0053] An aspect of the invention provides a method for assaying a compound which modulates the activity of a G protein-coupled receptor, comprising: isolating and mutating a nucleic acid molecule encoding a wild-type G protein-coupled receptor, wherein mutating the nucleic acid molecule comprises creating at least one nucleotide mutation resulting in an amino acid change in the wild-type G protein-coupled receptor, such amino acid being expressed in transmembrane domain III or VI, thereby producing a second nucleic acid molecule sequence encoding a mutant receptor; separately assaying the basal activity of the wild-type receptor, the mutant receptor and a negative control; determining whether the activity of the mutant receptor is greater than the wild-type receptor and the negative control; and if the basal activity of the mutant receptor is greater, then assaying the compound at the mutant receptor to determine whether the compound is an agonist, inverse agonist or antagonist of the G protein-coupled receptor.

[0054] In one aspect of the invention, the wild-type G protein-coupled receptor comprises human GPR88, or variants thereof. The variant has at least 70%, such as 75%, 80%, 85% or 90% identity to the amino acid sequence of human GPR88, preferably at least 95%, such as 96%, 97%, 98% or even 99% identity to the amino acid sequence of human GPR88. In another aspect of the invention, the wild-type G protein-coupled receptor comprises SEQ ID NO: 2.

[0055] In other aspects of the invention, the amino acid change is expressed at the 25th amino acid within transmembrane domain III of the wild-type G protein-coupled receptor. In another aspect of the invention, the amino acid change is expressed at the 2nd, 3rd, 8th, or 12th amino acid within transmembrane domain VI of the wild-type G protein-coupled receptor.

[0056] In a further aspect of the method, the amino acid change in transmembrane domain III of the wild-type G protein-coupled receptor comprises an amino acid change of asparagine (N) to alanine (A). In one embodiment of the method, the amino acid change in transmembrane domain

III of the wild-type G protein-coupled receptor comprises an amino acid change of asparagine (N) to alanine (A) at or around amino acid position 137.

[0057] In one embodiment of the method, the amino acid change in transmembrane domain III of the wild-type G protein-coupled receptor comprises an amino acid change of asparagine (N) to alanine (A) at amino acid position 137 of SEQ ID NO: 2. In a particular embodiment the variant comprises further amino acid substitutions, deletions, and/or insertions of amino acids of SEQ ID NO:2 outside the transmembrane domains, in particular outside transmembrane domains III and VI.

[0058] In another aspect of the method, the amino acid change in transmembrane domain VI of the wild-type G protein-coupled receptor comprises an amino acid change selected from the group consisting of glycine (G) to histidine (H), glycine (G) to glutamic acid (E), glycine (G) to lysine (K), leucine (L) to histidine (H), leucine (L) to glutamic acid (E), leucine (L) to lysine (K), leucine (L) to phenylalanine (F), leucine (L) to glutamic acid (E), phenylalanine (F) to valine (V), phenylalanine (F) to leucine (L), and phenylalanine (F) to alanine (A).

[0059] In another embodiment, the amino acid change in transmembrane domain VI of the wild-type G protein-coupled receptor comprises an amino acid change selected from the group consisting of glycine (G) to histidine (H) at or around amino acid position 283, glycine (G) to glutamic acid (E) at or around amino acid position 283, glycine (G) to lysine (K) at or around amino acid position 283, leucine (L) to histidine (H) at or around amino acid position 284, leucine (L) to glutamic acid (E) at or around amino acid position 284, leucine (L) to lysine (K) at or around amino acid position 284, leucine (L) to phenylalanine (F) at or around amino acid position 289, leucine (L) to glutamic acid (E) at or around amino acid position 289, phenylalanine (F) to valine (V) at or around amino acid position 293, phenylalanine (F) to leucine (L) at or around amino acid position 293, and phenylalanine (F) to alanine (A) at or around amino acid position 293.

[0060] In an embodiment of the invention, the amino acid change in transmembrane domain VI of the wild-type G protein-coupled receptor comprises an amino acid change at amino acid position 283, amino acid position 284, amino acid position 289 or amino acid position 293 of SEQ ID NO:2.

5

[0061] As used herein, an amino change which occurs within a specified transmembrane domain, for example, within TMIII or TMVI of the wild-type G protein-coupled receptor is counted from the beginning of the transmembrane region. Transmembrane domains of the GPR88 receptor are indicated by underlining the amino acids included in such TM regions as in Figure 2. As such, the first amino acid within TMI is arginine (R), followed by the 2nd amino acid isoleucine (I), and the 3rd amino acid proline (P), and so forth, with the 26th amino acid being valine (V) in TMI as depicted in Figure 2.

10

[0062] As used herein, an “amino acid change at or around amino acid position” means an amino acid within 3 amino acid positions upstream or downstream of the position number of the polypeptide, such position number being counted from the start codon (methionine) (M) of the polypeptide. The amino acid change at or around an amino acid position number may be within 3 amino acids, 2 amino acids or 1 amino acid upstream or downstream from the specified position number of the polypeptide, for example, from position number 137, 283, 284, 289 or 293 of the human GPR88 polypeptide of SEQ ID NO: 2.

15

20

[0063] In another embodiment of the method, the nucleic acid molecule encoding a mutant receptor comprises a nucleic acid molecule having a nucleotide sequence selected from the group consisting of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26. In more embodiments, the mutant receptor comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27.

25

[0064] In particular embodiments, the method further comprises determining whether the compound is an inverse agonist or partial agonist. The method may further comprise determining whether the compound is a neutral antagonist, in the presence of an agonist.

5 [0065] In other embodiments, the wild-type receptor is a GPR88 receptor. In other embodiments, the wild-type receptor is a human GPR88 receptor. In particular embodiments, the wild-type receptor comprises SEQ ID NO: 2.

10 [0066] In more embodiments, the wild-type and mutated nucleic acid molecules comprise cDNA.

[0067] In another embodiment, the negative control consists of empty vector control or a vector comprising a nonconstitutively active receptor nucleotide sequence.

15 [0068] In another embodiment of the invention, assaying the basal activity of the receptors is done in the absence of a receptor ligand; or alternatively, assaying the basal activity of the receptors is done in the presence of a receptor agonist or antagonist.

20 [0069] In further embodiments, assaying the basal activity of the receptors comprises transfecting a first host cell with a nucleic acid molecule encoding a wild-type receptor, a second host cell with a nucleic acid molecule encoding a mutant receptor, and a third host cell with a negative control. In another embodiment, the first and second host cells are cotransfected with a reporter construct comprising a promoter operably linked to the wild-type receptor or the mutant receptor.

25 [0070] In some embodiments, the promoter comprises a response element. In some embodiments, the response element is sensitive to a signal induced by the receptor. In other embodiments, the receptor induces a signal via a G protein selected from $G\alpha_q$, $G\alpha_i$, or $G\alpha_s$. In another embodiment, the receptor induces a signal via a $G\alpha_q5i$. In other embodiments, the
30 response element is selected from a somatostatin promoter, serum response element, cAMP

response element, NFκB response element, NFAT response element, and AP-1 response element.

[0071] In still other embodiments, the reporter construct comprises a reporter gene encoding a reporter molecule. In other embodiments, the reporter construct produces a reporter molecule. In more embodiments, the reporter molecule is luciferase, beta-galactosidase, or chloramphenicol acetyl transferase (CAT). In other embodiments, the reporter molecule is a transcriptional reporter molecule. In another embodiment, the reporter molecule is a luciferase polypeptide comprising SEQ ID NO: 30.

[0072] In particular embodiments, the reporter gene is a modified reporter gene comprising SEQ ID NO: 28.

[0073] In another embodiment, the reporter construct comprises SEQ ID NO: 28. In another embodiment, the reporter construct encodes a polypeptide having the amino acid sequence of SEQ ID NO: 29. In another embodiment, the expressed reporter gene is a luciferase polypeptide comprising SEQ ID NO: 29. In another embodiment, the expressed reporter gene is a luciferase polypeptide comprising SEQ ID NO: 30. In another embodiment, the luciferase reporter gene has the nucleotide sequence shown in SEQ ID NO: 28.

[0074] In other embodiments, the assay is a transcriptional reporter assay. In still other embodiments, the assay is a transcriptional reporter assay selected from a cAMP, luciferase reporter, beta-galactosidase reporter, chloramphenicol acetyl transferase reporter, intracellular calcium measurement, mitogenesis, GTPγS, MAP kinase activity, inositol phosphate, arachidonic acid release, or extracellular acidification rate assay.

[0075] In another embodiment of the invention, the basal activity of the mutant receptor is at least 25% greater than the basal activity of the wild-type receptor; or, in still another embodiment, the basal activity of the mutant receptor is at least 50% greater than the basal activity of the wild-type receptor.

[0076] In even other embodiments, the basal activity of the mutant receptor is at least 75%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or 1000% greater than the basal activity of the wild-type receptor; or even more than 1000% greater than the basal activity of the wild-type receptor.

[0077] In some embodiments of the invention, the nucleic acid molecule encoding the mutant receptor comprises at least 10, 15, 20, 25 or 50 consecutive nucleotides of SEQ ID NO: 3. In more embodiments, the nucleic acid molecule encoding the wild-type receptor comprises at least 10, 15, 20, 25 or 50 consecutive nucleotides of SEQ ID NO: 1.

[0078] In other embodiments, variants of the nucleotides and polypeptides of the invention may be used in the above methods.

[0079] Another aspect of the invention provides for a method for detecting or measuring G protein-coupled receptor activity comprising: cotransfecting a host cell with an expression vector comprising a promoter operably linked to a nucleic acid molecule encoding a constitutively active mutant G protein-coupled receptor, wherein the receptor can induce a ligand-independent intracellular signal, and a reporter construct comprising a response element operably linked to a reporter gene, wherein the reporter gene is a modified reporter gene that is rapidly expressed by the intracellular signal induced by the receptor, and detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof within 24 hours of incubation time following the cotransfection of the expression vector and reporter construct.

[0080] In one aspect of the invention, the amount of the expressed reporter gene is detectable by use of an assay that directly or indirectly measures the activity of the polypeptide encoded by the expressed reporter gene.

[0081] In one aspect of the method, detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof is by means of measuring the level of activity that the

expressed reporter gene has on an enzyme substrate. In further aspects, detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof may be done within 4 hours, or within 5 hours, or within 6 hours, or within 7, 8, 9, 10, 11, or 12 hours.

5 [0082] In some embodiments of the invention, the response element is selected from a somatostatin promoter, serum response element, cAMP response element, NFκB response element, NFAT response element, and AP-1 response element. In other embodiments, the reporter construct comprises a luciferase reporter gene, beta-galactosidase reporter gene, or chloramphenicol acetyl transferase reporter gene.

10

[0083] In particular embodiments, the reporter gene is a modified reporter gene comprising SEQ ID NO: 28.

15 [0084] In still other embodiments, the method further comprises cotransfecting the host cell with a chimeric G protein. In some embodiments, the chimeric G protein is selected from Gq5i, Gq5o, Gq5z, Gq5s, Gs5q, and G135z.

[0085] In more embodiments, the method further comprises cotransfecting the host cell with an
20 expression vector encoding a transactivating factor, which activates the reporter construct. In still more embodiments, the method further comprises cotransfecting the host cell with an expression vector encoding the activation domain of a transactivating factor. In still more embodiments, the method further comprises cotransfecting the host cell with an expression vector encoding the DNA binding domain of a first transactivating factor and an activation domain of a second
25 transactivating factor.

[0086] In another embodiment, the reporter construct comprises a reporter gene and a promoter containing a GAL4 upstream activating sequence (UAS), or any promoter sufficient to direct transcription. In another embodiment, the transactivating factor comprises a Gal4 DNA binding
30 domain and CREB activation domain.

[0087] In another embodiment, the reporter construct comprises SEQ ID NO: 28. In another embodiment, the reporter construct encodes a polypeptide having the amino acid sequence of SEQ ID NO: 29. In another embodiment, the expressed reporter gene is a luciferase polypeptide comprising SEQ ID NO: 29. In another embodiment, the expressed reporter gene is a luciferase polypeptide comprising SEQ ID NO: 30. In another embodiment, the luciferase reporter gene has the nucleotide sequence shown in SEQ ID NO: 28.

[0088] In more embodiments, the host cell is contacted with a test compound or test ligand. In another embodiment, an increased amount of expressed reporter gene is detected in the host cell contacted with a test compound or test ligand compared to a host cell that is not contacted with a test compound or test ligand. In further embodiments, a decreased amount of expressed reporter gene is detected in the host cell contacted with a test compound or test ligand compared to a host cell that is not contacted with a test compound or test ligand.

[0089] In another aspect of the invention, the nucleic acid molecule comprises SEQ ID NO:3. Another aspect of the invention provides a constitutively active mutant G protein-coupled receptor comprising a polypeptide encoded by SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26.

[0090] Another aspect of the invention provides a constitutively active mutant G protein-coupled receptor comprising a polypeptide having the amino acid sequence of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 or 27.

[0091] Another aspect of the invention provides an isolated nucleic acid molecule comprising SEQ ID NO: 3.

[0092] Another aspect of the invention provides an isolated nucleic acid molecule comprising SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26; or vectors comprising any of the

isolated nucleic acid molecules of the invention; or cells transfected with any of the isolated nucleic acid molecules or the vectors of the invention.

5 [0093] Another aspect of the invention provides an isolated nucleic acid molecule which is complementary to SEQ ID NO:3. Other aspects of the invention provide an isolated nucleic acid molecule which is complementary to any of the nucleotides of the invention.

10 [0094] Another aspect of the invention provides an isolated nucleic acid molecule encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, or variants thereof.

15 [0095] Another aspect of the invention provides an isolated polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, or variants thereof. In a particular embodiment the variant comprises further amino acid substitutions, deletions, and/or insertions of amino acids of SEQ ID NO:2 outside the transmembrane domains, in particular outside transmembrane domains III and VI.

20 [0096] A further aspect of the invention provides a fragment of the polypeptides of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, wherein the fragment comprises an amino acid sequence having at least 293 contiguous amino acids. A further aspect of the invention provides a fragment of the polypeptides of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 or 27, wherein the fragment comprises an amino acid sequence having at least 50 contiguous amino acids.

25 [0097] A further aspect of the invention provides a fragment of SEQ ID NO: 5 comprising histidine (H) at amino acid position 283. An aspect of the invention provides a fragment of SEQ ID NO: 7 comprising lysine (K) at amino acid position 284. An aspect of the invention provides a fragment of SEQ ID NO: 9 comprising glutamic acid (E) at amino acid position 284. An aspect of the invention provides a fragment of SEQ ID NO: 11 comprising glutamic acid (E) at amino acid position 283. An aspect of the invention provides a fragment of SEQ ID NO: 13 comprising

30

lysine (K) at amino acid position 283. An aspect of the invention provides a fragment of SEQ ID NO: 15 comprising histidine (H) at amino acid position 284. An aspect of the invention provides a fragment of SEQ ID NO: 17 comprising alanine (A) at amino acid position 293. An aspect of the invention provides a fragment of SEQ ID NO: 19 comprising leucine (L) at amino acid position 293. An aspect of the invention provides a fragment of SEQ ID NO: 21 comprising valine (V) at amino acid position 293. An aspect of the invention provides a fragment of SEQ ID NO: 23 comprising glutamic acid (E) at amino acid position 289. An aspect of the invention provides a fragment of SEQ ID NO: 25 comprising phenylalanine (F) at amino acid position 289. A further aspect of the invention provides a fragment of SEQ ID NO: 27 comprising alanine (A) at amino acid position 137.

[0098] Another aspect of the invention provides an isolated nucleic acid encoding any of the polypeptides of the invention, or variants thereof.

[0099] The term "variant" may be used to refer to an oligonucleotide sequence which differs from the related wild-type sequence in one or more nucleotides. Such a variant oligonucleotide is expressed as a protein or polypeptide or receptor "variant" which, as used herein, indicates a polypeptide sequence that differs from the wild-type polypeptide by one or more mutations, i.e. in the substitution, insertion or deletion of one or more amino acids. In one embodiment the protein variant comprises at most 30 mutations, such as 25 mutations or less, e.g. 20 mutations, 15 mutations, 10, mutations or less, such as 9, 8, 7, 6, 5, 4, 3, or 2 mutations or a single mutation. In a preferred embodiment not more than 6, such as 5, 4, 3, or 2 mutations or a single mutation is in the transmembrane domain of the receptor variant, preferably in transmembrane domain III or VI. The variant polypeptide differs in primary structure (amino acid sequence), but may or may not differ significantly in secondary or tertiary structure or in function relative to the wild-type. As such, the receptor activity of the variant receptor is not significantly altered as compared to the wild-type receptor. In the context of the present invention, amino acid positions of variants of SEQ ID NO: 2 are defined by alignment of the variant with SEQ ID NO: 2, and the corresponding amino acid position number of SEQ ID NO: 2 is used to define the amino acid position number of the variant.

[00100] A “functionally conservative mutation” as used herein intends a change in a polynucleotide encoding a variant polypeptide in which the activity is not substantially altered compared to that of the polypeptide from which the variant is made. Such variants may have, for example, amino acid insertions, deletions, or substitutions in the relevant molecule that do not substantially affect its properties, or as used in this context, do not substantially alter the receptor activity. For example, the variant, or functionally conservative mutant, can include conservative amino acid substitutions, such as substitutions which preserve the general charge, hydrophobicity/hydrophilicity, side chain moiety, and/or steric bulk of the amino acid substituted, for example, Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, Thr/Ser, and Phe/Trp/Tyr. As such, variants of the wild-type receptors of the invention may not have substantially altered basal activity, until a mutation is introduced according to the methods of the invention, thereby creating a constitutively active receptor.

[00101] “Structurally conservative mutant” as used herein means a polynucleotide containing changes in the nucleic acid sequence but encoding a polypeptide having the same amino acid sequence as the polypeptide encoded by the polynucleotide from which the degenerate variant is derived. This can occur because a specific amino acid may be encoded by more than one “codon,” or sequence of three nucleotides. For a typical protein sequence of 300 amino acids there are over 10^{150} codon combinations that will encode an identical protein. As used herein, structurally conservative mutants may be nucleotides having 30%, or 25%, or 20%, 15%, or 10% of their nucleic acids changed but encoding the same polypeptide as the wild-type polypeptide from which it was derived. The integrity of a variant sequence may be tested by determining the level of expression of the polypeptide in a host cell by techniques well-known in the art, such as immunochemistry or an assay that measures receptor activity. As such, the protein expression level of the variant receptor is not significantly altered as compared to the wild-type. In the context of the present invention, SEQ ID NO: 3 encodes for a human GPR88 receptor with an equivalent level of protein expression in a host cell compared to a wild-type human GPR88 receptor.

[00102] In some embodiments, the mutant receptor is encoded by a nucleotide having 70%, 75%, 80%, 85%, 90% or 95% nucleic acid identity to SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, and 27.

[00103] For the purpose of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends in Genetics* 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

[00104] For purposes of the present invention, the degree of identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, 2000, *supra*) preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4). The output of Needle labeled “longest identity” (obtained using the nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

[00105] Compounds that bind to, activate or inhibit the polypeptides of the invention, thereby increasing or decreasing GPR88 receptor activity, are potential therapeutic drug candidates. The present invention provides methods to identify therapeutic compounds useful for the treatment of a subject suffering from a disease or disorder, such as Parkinson’s disease, Huntington’s disease, restless leg syndrome or other movement disorders, schizophrenia, drug addiction, and epilepsy.

Accordingly a therapeutically effective amount of the identified compound is administered to the subject in need of such treatment.

[00106] The methods of the present invention involve mutating a gene or a nucleic acid sequence encoding a wild-type receptor to produce a nucleic acid sequence which encodes a mutated receptor. The gene or nucleic acid may be cDNA, genomic DNA, or RNA with the understanding that the mutation would ultimately result in expression of the receptor. Preferably, the mutations are induced in region(s) of the nucleic acid encoding the wild type receptor to result in a mutated receptor having increased basal activity relative to the wild type receptor. Preferably, the increased activity is in the absence of a receptor ligand. The mutated nucleic acid is then introduced into a cell, and the mutated receptor is expressed, preferably on the cell surface. An assay is then carried out to detect an alteration in signaling of the mutant receptor compared to the wild-type receptor, and/or a negative control. An increase in signaling in the mutant receptor, compared to the wild-type receptor or negative control, identifies the mutant receptor is constitutively active, and the mutated receptor may be suitable for further assay development. The activity of the receptors may be measured by assaying for molecules produced in a signaling pathway. Signaling by the receptor can be ligand dependent or independent signaling. In one embodiment, the receptor with altered signaling can be further screened for an alteration in a response induced by a ligand, which may be a drug, an agonist, a partial agonist, an antagonist, or an inverse agonist.

[00107] Thus “receptor activation” or “receptor activity” as used herein means producing an active state of a G-protein coupled receptor which then transduces a signal through the cell, usually via a G protein interacting with such receptor. G proteins thus interact with second messenger systems in the cell to further transduce this signal. Intracellular signal transduction events are well understood by the skilled person and responsible for many downstream effects, e.g. transcription, morphogenesis, apoptosis, or activation of many other cellular events. Second messenger systems activated by G proteins are well known in the art and can be measured in many ways depending on the G protein pathway that is activated, thus detecting receptor activity. Examples of second messenger assays used to detect receptor activity include, but are not limited to, measurements of cyclic AMP (cAMP) levels, intracellular calcium mobilization, mitogen-activated pathway kinase (MAPK) activity, inositol phosphate accumulation, arachidonic acid

release, GTP γ S function, cell metabolism by microphysiometry, cell proliferation, chloride currents and potassium currents. Expression of the receptors of the present invention in a host cell line can result in the activation of the second messenger responses described above, and a wide spectrum of assays can be used to screen for agonists, partial agonists, inverse agonists and antagonists of the receptor.

[00108] As an alternative to measuring molecules in a signaling pathway directly to identify constitutively active receptors, a reporter assay system may be utilized in which a response element, responsive to signaling through a particular receptor, is attached to a reporter gene in combination with a transcriptional promoter. Specifically, the expression of the reporter gene is controlled by the activity of the receptor. This method involves the steps of 1) identifying a response element that is sensitive to signaling by a specific receptor, 2) operably linking the response element and a promoter to a reporter gene. Thus, the mutated receptor produces a response which interacts with the response element, which in turn activates the promoter. The basal level reporter activity of a putative mutated receptor may then be compared to the wild-type receptor or negative control. A constitutively active receptor exhibits a statistically significant increase in basal activity, e.g. at least about a 25%, 50%, 75%, 100%, or an even greater than 1000% increase in basal activity, when compared to the wild-type receptor or negative control.

[00109] In another embodiment, the reporter assay further includes a chimeric G protein capable of switching the signaling of the receptor to a different pathway than provided by the wild-type receptor's endogenous G protein-coupling. The chimeric G protein includes a G protein with the C-terminal amino acids changed to those of another G protein. In one embodiment, the chimeric G protein can be the chimeric G protein, Gq5i. In another embodiment, the chimeric G protein can be Gq5i, Gq5o, Gq5z, Gq5s, Gs5q, or G135z.

[00110] Assays useful in the present invention include any assay which may be used to detect protein signaling, and includes one or more response elements. One of skill in the art would recognize that the response element used in the present assay can be any response element that is sensitive to signaling through the receptor. For example, for reporter assays coupled to G proteins, one would select responses elements that are sensitive to signaling through a G-protein

coupled receptor. Examines of such response elements may include a portion of the somatostatin (SMS) promoter, the serum response element (SRE), the cAMP response element (CRE), NFAT response element and AP-1 response element. For example, SMS is activated by coupling of GPCRs to either $G\alpha_q$ or $G\alpha_s$; the serum response element (SRE) is activated by receptor coupling to $G\alpha_q$; the cAMP response element (CRE) is activated by receptor coupling to $G\alpha_s$ and inhibited by coupling to $G\alpha_i$; and the TPA response element (sensitive to phorbol esters) is activated by receptor coupling to $G\alpha_q$; and the NF κ B element is activated by receptor coupling to $G\alpha_i/o$, $G\alpha_q$ or $G\alpha_s$. Each of these response elements can be employed in a reporter assay to determine the basal level activity of a G protein-coupled receptor.

[00111] In addition, a reporter construct for detecting receptor signaling might include a response element that is a promoter sensitive to signaling through a particular receptor. For example, the promoters of genes (e.g., encoding epidermal growth factor, gastrin, or fos) can be operably linked to a reporter gene for detection of G protein-coupled receptor signaling. The reporter construct can be a luciferase construct, a beta-galactosidase construct, or a chloramphenicol acetyl transferase construct.

[00112] Other response elements include response elements sensitive to signaling through a single transmembrane receptor or a nuclear receptor.

[00113] The signaling detected by the particular response element can be any receptor signaling, including increased basal signaling (constitutive signaling), decreased basal signaling (silencing), and hypersensitive as well as hyposensitive signaling.

[00114] In other embodiments of the invention, the G protein-coupled receptor can be a constitutively active receptor, a hypersensitive receptor, or a hyposensitive receptor. In other embodiments of the invention, the G protein-coupled receptor can be coupled to a G protein, for example, $G\alpha_q$, $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, $G\alpha_z$ or $G\alpha_{13}$. In another embodiment, a third expressing vector including a promoter operably linked to transactivating factor, which activates the reporter construct may be included. This type of construct is typically included to confer a higher degree of specificity toward activation of a reporter through a specific pathway. Examples of this type of construct can be found in Stratagene's PathDetection system (see, e.g., the Stratagene catalog:

PathDetectB in Vivo Signal Transduction Pathway cis-Reporting Systems Introduction Manual or PathDetectB in Vivo Signal Transduction Pathway trans-Reporting Systems Introduction Manual, Stratagene, La Jolla, CA).

5 [00115] The present invention also provides for the detection of alterations in the signaling activity of a receptor. In one preferred embodiment, the screening assay is used to detect alterations in the basal level of signaling of a receptor, e.g., a mutated or wild-type receptor. Receptors with increased basal level signaling are identified as constitutively active receptors. Constitutively active receptors include constitutively active G protein-coupled receptors (e.g., opiate receptors), single transmembrane domain receptors (e.g., the erythropoietin receptor (EPO
10 receptor)), and nuclear receptors (e.g., steroid hormone receptors, such as the estrogen receptor). In embodiment, the screening assay may also be used to detect a decrease in the basal level signaling of a particular (e.g., naturally occurring constitutively active) receptor, for example, receptors having silencing mutations.

15 [00116] According to the present invention, constitutively active receptors with increased basal activity are compared to the appropriate negative control. For example, naturally occurring, or endogenous, constitutively active receptor, exhibit an increased basal level of signaling as compared to the activity of an expressed vector lacking a gene encoding a receptor.

[00117] Alternatively, mutant receptors, or non-endogenous receptors, having constitutive
20 active receptor to the basal level activity of the wild-type receptor. A statistically significant increase in basal level activity in a candidate receptor compared to a control or wild-type receptor indicates identification of a constitutively active receptor. Many naturally occurring (endogenous) and non-naturally occurring (non-endogenous) constitutively active receptors have been previously identified and are available in the art. As described herein, this information can
25 be harnessed and used as a tool to identify additional constitutively active receptors. Preferably, an increase in basal level activity is detected by measuring an increase in basal level signaling in the mutant receptor, compared to the wild-type receptor.

[00118] The person skilled in the art will appreciate that any assay typically used for measuring the ligand-stimulated activity of the wild-type receptor may also be used to measure the basal level activity of a mutant receptor. Such assays are discussed in further detail herein, below.

[00119] The present invention provides a method of identifying constitutively active receptors.

5 As noted above, some receptors (e.g., wild-type receptors) are naturally constitutively active. Such naturally occurring constitutively active receptors are identified by simply comparing the basal activity of the wild-type receptor to that of a negative control.

[00120] A suitable negative control is, for example, a cell lacking expression of the natural wild-type receptor (e.g., a cell transfected with an empty expression vector), a cell transfected with a
10 wild-type receptor, or a cell transfected with a different receptor that has been previously established to lack constitutive activity (preferably both an empty expression vector and a wild-type vector are used). Alternatively, the present invention provides a method of identifying mutation-induced constitutively active receptors.

[00121] According to the present invention, mutation-induced constitutively active receptors
15 may be identified systematically by (1) identifying regions of homology between a nonconstitutively active wild-type receptor and one or more constitutively active receptors; (2) introducing mutations into one or more regions of the nonconstitutively active, e.g., wild-type receptor, based on the identified region(s) of homology; and (3) assaying the mutant receptors for constitutive activity. Methods of achieving each of these steps are described in detail below.

20 One skilled in the art will appreciate that the mutations can be introduced by any random mutagenesis procedure standard in the art. A large variety of random mutagenesis kits are in fact commercially available. Once identified, the constitutive activity of the receptor may be confirmed, for example, using a mammalian expression system, or a yeast expression system.

[00122] Numerous constitutively active receptors (naturally occurring and non-naturally
25 occurring) have been previously identified. Such receptors provide information that can be used to identify additional constitutively active receptors. To complete step (1), above, available nucleic acid and for amino acid sequence information, preferably amino acid sequence information, including wild-type and mutant receptors, is compiled to generate a database of

constitutively active receptor sequences. Next, the sequence of a given nonconstitutively active receptors of therapeutic interest (e.g., a receptor known to be a receptor for an agonist) is compared to the many sequences of constitutively active receptors in the database to identify regions that are conserved between the nonconstitutively active receptor and the one or more
5 constitutively active receptors.

[00123] Specific residues in the nonconstitutively active wild-type receptor are targeted for mutation based on the identified regions of homology between the nonconstitutively active receptor and constitutively active receptor(s), which are likely to impart constitutive activity onto the nonconstitutively active receptor (see, e.g., Fig. 1). For example, to complete step (2), above,
10 if a region of homology between a nonconstitutively active receptor and a constitutively active receptor is identified that is conserved in all amino acids but one, a mutation is introduced into the nonconstitutively active receptor to make the conserved region in the nonconstitutively active receptor identical to that of the constitutively active receptor.

[00124] Alternatively, if the region conserved between the nonconstitutively active receptor and
15 the constitutively active receptor shows a high degree of amino acid similarity, a series of targeted mutations are introduced into the nonconstitutively active receptor that are likely, based on the degree of homology and the knowledge of the skilled artisan, to make the receptor constitutively active. As for another example, the nonconstitutively active receptor might share a region of homology with another nonconstitutively active receptor that has been made
20 constitutively active by the introduction of a certain mutation or mutations. In this case, the same or similar mutations are introduced into the given nonconstitutively active receptor. Alternatively, the database is used to identify regions of homology between a naturally occurring receptor of therapeutic interest and one or more constitutively active receptors. The identified regions of homology would lead the skilled artisan to test the naturally occurring receptor for
25 constitutive activity.

[00125] Step (3) involves assaying the mutant receptors for constitutive activity by assaying for an increase in basal activity of the receptor. In one embodiment of the present invention, a reporter assay system in which a response element, responsive to signaling through a particular receptor, is attached to a reporter gene in combination with a transcriptional promoter. Thus, the

expression of the reporter gene is controlled by the activity of the chosen receptor. This method involves the steps of (a) identifying a response element that is sensitive to signaling by a specific receptor polypeptide (e.g., by eliciting an increase or decrease in gene expression upon receptor activation); (b) operably linking the response element and a promoter (if the promoter is not included in the response element) to a reporter gene; and (c) comparing the basal level reporter activity of a putative constitutively active receptor to a negative control, an increase in basal level reporter activity compared to the negative control indicating the identification of a constitutively active receptor. Preferably the increase in basal activity is at least 25%, 50%, 75%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or 1000% over the basal activity of the negative control. In preferred embodiments, this assay system is used to screen for receptor mutants exhibiting constitutive activity. The receptor can be any receptor identified as a candidate constitutively active receptor.

[00126] A wide variety of reporter constructs can be generated that are sensitive to any of a variety of signaling pathways induced by signaling through a particular receptor (e.g., a second messenger signaling pathway). Additional response elements, including promoter elements, can be found in the Stratagene catalog (PATHDETECT, PathDetectB in Vivo Signal Transduction Pathway cis-Reporting Systems Introduction Manual or PathDetectB in Vivo Signal Transduction Pathway trans-Reporting Systems Introduction Manual, Stratagene, La Jolla, CA).

[00127] In one embodiment, a G protein-coupled reporter assay system includes a reporter construct containing a response element that is sensitive to signaling through a specific G protein, and a promoter, operably linked to a reporter gene, optionally in combination with an expression vector containing a promoter operably linked to a nucleic acid encoding a receptor, wherein the receptor is coupled to a G protein, or other downstream mediator, to which the selected response element is sensitive.

[00128] The present invention includes use of specific response elements that are sensitive to signaling through each of $G\alpha_q$, $G\alpha_s$, and $G\alpha_i$. In addition, detection of a ligand-stimulated decrease in intracellular cAMP relies on whether a large enough percentage of the cells are successfully transfected with, and express, the receptor molecule.

[00129] In order to measure a decrease in the signaling from reporter based assay it is important that the stability of the reporter protein is not too high, e.g., when measuring the activity of a luciferase protein it is important that the half-life is short, e.g., about 30 minutes, 60 minutes, 90 minutes, or 120 minutes. In one embodiment of the present invention, a reporter construct expresses a luciferase protein (SEQ ID NO: 29) with a rapid turnover, i.e., to increase the response rate of the signaling from the receptor. The invention provides an optimized luciferase construct which increases the response rate for the signaling of the receptor. In one embodiment, the luciferase construct comprises SEQ ID NO: 28. Advantages of the optimized luciferase construct include shortened incubation time.

[00130] To measure the activity of a G-protein coupled receptor which is $G\alpha_i$ coupled, it is often desirable or necessary to co-express a chimeric G-protein in order to drive the coupling toward a pathway where a response can be measured. $G\alpha_i$ coupling may be detected by altering the signaling pathway generated by $G\alpha_i$ coupled receptors. For example, a chimeric G protein that contains the entire $G\alpha_q$ protein having the five C-terminal amino acids from $G\alpha_i$ attached to the C-terminus of $G\alpha_q$ has been generated (Holst et al., 2003, *Molecular Endocrinology* 17(11):2201–2210; Broach et al., 1996, *Nature* 384 (Suppl.): 14- 16). The chimeric G protein, e.g. $G\alpha_{qi5}$, is recognized as $G\alpha_i$ by $G\alpha_i$ coupled receptors, but switches the receptor induced signaling from $G\alpha_i$ to $G\alpha_q$. This allows $G\alpha_i$ receptor coupling to be detected using a positive assay by use of the $G\alpha_q$ responsive CRE-Luc construct (Stratagene, La Jolla, CA).

[00131] Moreover, any other chimeric G protein can be constructed by replacing or adding at least 3 amino acids, usually at least 5 amino acids, from the carboxyl terminus of a G protein (e.g., $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, $G\alpha_z$, or $G\alpha_o$) to a second G protein (e.g., $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, $G\alpha_z$, or $G\alpha_o$) which is either full-length or includes at least 50% of the amino terminal 25 amino acids. Various species homologs of G alpha proteins may be utilized according to the invention. See also U.S. Patent No. 7,067,277 B1, issued on June 27, 2006, the contents of which are hereby incorporated by reference in its entirety. Generally, the carboxyl-terminus of the $G\alpha$ protein subunit is a key determinant of receptor specificity.

[00132] For example, the G α _q can be made to respond to G α _i coupled receptors by replacing its carboxyl-terminus with the corresponding Gi2 alpha, Go, or Gz alpha residues. In addition, C-terminal mutations of Gq alpha/Gi alpha chimeras show that the critical amino acids are in the -3 and -4 positions, and exchange of carboxyl-termini between Gq alpha and Gs alpha allows
5 activation by receptors appropriate to the C-terminal residues. Furthermore, replacement of the five carboxyl-terminal amino acids of Gq alpha with the Gs alpha sequence permitted certain Gs alpha-coupled receptors (the V2 vasopressin receptor, but not the beta 2-adrenoceptor) to stimulate phospholipase C. Replacement of the five carboxyl-terminal amino acids of Gs alpha with residues of Gq alpha permitted certain Gq alpha-coupled receptors (bombesin and V1a
10 vasopressin receptors, but not the oxytocin receptor) to stimulate adenylyl cyclase. Thus, the relative importance of the G alpha carboxyl-terminus for permitting coupling to a new receptor depends on the receptor with which it is paired. Any other G protein chimera that is capable of switching the signaling from one G-protein coupled receptor to another pathway can also be used according to the invention.

[00133] In one embodiment, the constitutively active receptors identified by the screening assays of the present invention are used as tools for identifying ligands of a given receptor, including peptide, non-peptide, and small molecule ligands. For example, ligands (e.g., a hormone or a drug) that bind a particular constitutively active receptor may be identified using a reporter assay
15 system by (1) operably linking a response element, which is sensitive to receptor activation, and a promoter, to a reporter gene to generate a receptor activation sensitive reporter construct; (2) cotransfecting cells with the reporter construct and an expression vector containing nucleic acid encoding the constitutively active receptor; (3) contacting the cells with a ligand; and 4) assaying
20 for ligand-dependent activation or inhibition of the reporter construct, an increase or decrease in the ligand-dependent activation, compared ligand-independent signaling, indicating the presence of an agonist or inverse agonist, respectfully.
25

[00134] Furthermore, ligands (e.g., a hormone or a drug) that bind a particular constitutively active receptor may be identified using assays well-known to the person of skill in the art including, but not limited to cAMP, luciferase reporter, beta-galactosidase construct, chloramphenicol acetyl transferase construct, intracellular calcium measurement, mitogenesis,

GTP γ S, MAP kinase activity, inositol phosphate, arachidonic acid release, or extracellular acidification rate assays.

[00135] Ligands that activate or inhibit a particular receptor by increasing or decreasing receptor activity may, upon further experimentation, prove to be valuable therapeutic drugs for treatment of disease.

[00136] In another embodiment, when applied to constitutively active receptors (wild-type or mutant), a panel of reporter gene constructs that are sensitive to different signaling pathways (e.g., SRE-Luc, SMS-Luc, and CRE-Luc) can be used to predict the second messenger pathway that will be activated by the endogenous receptor ligand (e.g., cAMP, inositol phosphate production). This information will facilitate and accelerate both the identification of cognate endogenous ligands, and the discovery of drugs that act on receptors by the use of the inventive high-throughput screening based techniques.

[00137] The present invention also provides a mutant GPR88 receptor which is constitutively active compared to the wild-type GPR88 receptor. Specifically, the mutated GPR88 receptor may exhibit activity even in the absence of its ligand or antagonist. If wild-type GPR88 receptors have a degree of basal activity in the absence of its ligand or antagonist, the mutated GPR88 receptors exhibit from 25% to 2000% greater activity in the absence of its ligand or antagonist, i.e., has 25% to 2000% greater basal activity compared to the basal activity of the wild-type GPR88 receptor. Such receptors may include SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, or variants thereof. It is believed an amino acid substitution in at least one position in the membrane spanning region of the receptor protein causes the change of the activity of the receptor. These mutant receptors were tested using a transcriptional reporter assay capable of measuring the basal activity of the GPR88 receptor. Cells were cotransfected with a luciferase reporter construct containing a transcriptional response element and one of either the wild-type GPR88 receptor, a mutant GPR88 receptor, or a negative control. Cells were additionally be transfected with a transactivating factor construct, such as a construct containing both a Gal4 DNA binding domain and a CREB activating domain, as described hereinafter.

[00138] The present invention is illustrated by the following EXAMPLES. The foregoing and following description of the present invention and the various embodiments are not intended to be limiting of the invention but rather are illustrative thereof. Hence, it will be understood that the invention is not limited to the specific details of these EXAMPLES. For instance, those skilled in the art will understand and appreciate from these EXAMPLES, based on the present description, how to apply the methods of the invention to determine a method for assaying a compound which modulates the activity of a GPCR, and to use the disclosed nucleic acid molecules and polypeptides to identify therapeutic compounds.

EXAMPLES

[00139] Example 1 – Construction of Artificial GPR88 nucleotide sequence

[00140] The nucleotide sequence of human GPR88 is disclosed at GenBank accession no. AY336999, containing 1155 nucleotides (SEQ ID NO:1). The amino acid sequence of wild-type human GPR88 is a 384 amino acid sequence, GenBank accession no. AAQ76787 (SEQ ID NO: 2). The GPR88 DNA sequence is highly GC rich (> 75 % GC content), and is thus difficult to mutate by traditional mutagenesis techniques. Accordingly a structurally conservative mutant GPR88 (SEQ ID NO: 3) was designed by carefully changing many nucleotides, and thus changing the codons that encode for the appropriate amino acid, without significantly altering the activity of the receptor. The oligonucleotide of SEQ ID NO: 3 was then created by gene synthesis methods (MWG Biotech AG, Ebersberg, Germany) and ligated to a suitable vector. The resulting GPR88 mutant (SEQ ID NO: 3) has a GC content of about 50% and has a sequence suitable to ease the construction of additional mutants by traditional mutagenesis technologies. The mutated nucleotide sequence of SEQ ID NO: 3 has 75% nucleic acid identity compared to the wild-type GPR88 sequence (SEQ ID NO: 1). SEQ ID NO: 3 encodes for a human GPR88 receptor with an equivalent level of protein expression as compared to wild-type GPR88 (SEQ ID NO: 1) as measured by routine immunochemistry.

[00141] Example 2 – Mutation of Artificial GPR88 nucleotide sequences

[00142] An overview of sequence information for known constitutively active Class I G protein-coupled receptors was generated by compiling available information, and residues within

Class I G protein-coupled receptors that are important for constitutive activity were identified (see Fig. 1A). These amino acids are scattered over most of the transmembrane domains. However, specific amino acid positions at the bottom of transmembrane domain III and transmembrane domain VI are over represented with respect to reported constitutive active mutations, e.g. the amino acids in the 25th location within TMIII and the amino acids in the 2nd, 3rd, 8th, and 12th location within TMVI as shown in Fig. 1B and Fig. 2. Mutants of SEQ ID NO: 3 were created (Fig. 1B) by site directed mutagenesis resulting in at least one amino acid substitution (MWG Biotech AG, Ebersberg, Germany). Twelve representative nucleotide sequences containing at least one amino acid substitution are presented as SEQ ID NOs: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26. Mutants were constructed by gene synthesis and inserted into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA), and transfected into HEK293T cells. Transfection of HEK293T cells was performed using Lipofectamine (Invitrogen, Carlsbad, CA), under standard conditions as recommended by the manufacturer. Other transfection techniques known to the skilled artisan may be used.

[00143] SEQ ID NO: 4 encodes a mutant hGPR88 peptide designated G283H, SEQ ID NO: 5.

[00144] SEQ ID NO: 6 encodes a mutant hGPR88 peptide designated L284K, SEQ ID NO: 7.

[00145] SEQ ID NO: 8 encodes a mutant hGPR88 peptide designated L284E, SEQ ID NO: 9.

[00146] SEQ ID NO: 10 encodes a mutant hGPR88 peptide designated G283E, SEQ ID NO: 11.

[00147] SEQ ID NO: 12 encodes a mutant hGPR88 peptide designated G283K, SEQ ID NO: 13.

[00148] SEQ ID NO: 14 encodes a mutant hGPR88 peptide designated L284H, SEQ ID NO: 15.

[00149] SEQ ID NO: 16 encodes a mutant hGPR88 peptide designated F293A, SEQ ID NO: 17.

[00150] SEQ ID NO: 18 encodes a mutant hGPR88 peptide designated F293L, SEQ ID NO: 19.

[00151] SEQ ID NO: 20 encodes a mutant hGPR88 peptide designated F293V, SEQ ID NO: 21.

[00152] SEQ ID NO: 22 encodes a mutant hGPR88 peptide designated L289E, SEQ ID NO: 23.

[00153] SEQ ID NO: 24 encodes a mutant hGPR88 peptide designated L289F, SEQ ID NO: 25.

[00154] SEQ ID NO: 26 encodes a mutant hGPR88 peptide designated N137A, SEQ ID NO: 27.

[00155] Of particular interest were N137A (SEQ ID NO: 27), G283H (NO:5), L284K (SEQ ID NO:7), L284E (SEQ ID NO:9), L289E (SEQ ID NO: 23), L289F (SEQ ID NO:25), F293A (SEQ ID NO: 17) and F293L (SEQ ID NO:19) GPR88 mutants.

[00156] Example 3 - Assaying Mutant GPR88 receptors for Constitutive Activity

Cells of Example 2 were cultured in DMEM including Glucose/Glutamax/Na- pyruvate (GIBCO, Invitrogen, Carlsbad, CA), with 10% FBS and 1% P/S. Cells were transferred to DEMEM medium without P/S prior to transfection (alternatively) the cells may be washed once with DMEM without P/S just prior to transfection), and are dispensed into a 96 well plate, 25,000 cells/well.

[00157] The following constructs were assayed accordingly:

Construct	96-well – plasmid DNA amount [ng]
pFR-Luc (Stratagene)	50 ng
pFA2-CREB (Stratagene)	6 ng
Gq-i5	25 ng
pcDNA3.1 control vector or NK1 control or Wild-type hGPR88 or N137A or G283H or G283E or G283K or L284H or L284E or L284K or L289F or L289E or F293V or F293L or F293A	10 ng

[00158] The cells may also be transferred to a 384-well cell plate format (Costar 3707, white transparent TC coated plates) and amounts adjusted accordingly. Following transfection, cells

were cultured in media for two days and tested for luminescence. Medium was removed and cells were washed once using 100 µl PBS.

[00159] For detection of luminescence, the following substrates were also added according to the manufacturer's instructions:

- 5 [00160] Luciferase substrate: BRITELITE PLUS (PerkinElmer), or STEADYLITE PLUS (PerkinElmer); OPTIMEM transfection medium (Invitrogen); and HBSS buffer (including CaCl₂ / MgCl₂) (Gibco).

[00161] Luminescence was detected using a Wallac microbeta TriLux luminometer (PerkinElmer). Luminescence readings may be done with standard equipment known to the skilled artisan. The luminometer should be very sensitive, with a low background for this assay. Figure 4 indicates the relative luminescent readout (luciferase activity) for each mutant GPR88 receptor.

[00162] Results indicate that mutant GPR88 receptors G283H (SEQ ID NO: 5) and G283K (SEQ ID NO: 13) are constitutively active mutant receptors, demonstrating constitutive activity that is higher than the basal activity of the wild-type receptor.

[00163] The amount of plasmid (cDNA) used in the above transfection protocol was adjusted to determine a dose response relationship by varying the amount of receptor expression. Figure 5 shows the dose response relationship for G283H GPR88 (SEQ ID NO: 5) vs. wild-type GPR88 receptor where the mutant receptor maintains a higher basal activity level at all amounts of cDNA up to 30 ng, relative to wild-type GPR88 receptor. The assay was adjusted for medium throughput and high throughput conditions (384 well format). Under these conditions, G283H GPR88 displays a robust luminescence reading that is several-fold higher than the basal level activity of the wild-type receptor (Fig. 6).

[00164] Example 4 –Optimization of the Mutant Constitutively Activity GPR88 Receptor Assay

[00165] The reporter construct, pFR-Luc (PATHDETECT, Invitrogen) was modified by destabilizing the luciferase reporter gene (see Fig. 7). A luciferase nucleic acid molecule was destabilized by introducing an additional sequence at the 3' end (SEQ ID NO: 28). The new construct, KT-001, expresses a 591 amino acid luciferase polypeptide (SEQ ID NO: 29) comprising a carboxy-terminal end sequence of
5'-IAVNSHGFPPEVEEQAAAGTLPMSCAQESGMDRHPAACASARINV-3' (SEQ ID NO: 30).

[00166] The assay was optimized in order to reduce the incubation time of the cells, thus avoiding compromising the cells and the biological response of the cells. Since the luciferase protein is normally very stable, having a half-life of approximately 3 hours, a long incubation period would be needed in order to accurately measure increments of decreased signal, such as upon contacting the receptor with an antagonist. Also, prolonged incubation, such as > 6 hours or overnight, would have to take into account the stability of any compounds tested, as well as the cellular environment. Cells transfected with GPR88 G283H or control (pCIneo vector), and with pFR-Luc (PATHDETECT, Invitrogen) (Fig. 8A) compared with KT-001 (Fig. 8B), were incubated in the presence of cyclohexamide and luminescence measured every hour. As seen in Fig. 8, the cells transfected with KT-001 exhibit luminescent signal comparable to background signal in approximately 3 hours. The KT-001 construct expressing SEQ ID NO: 29 therefore has a half-life of less than 1 hour (Fig. 8B). Assay incubation time was determined to be optimal at 4 to 5 hours for this assay.

CLAIMS:

1. A method for assaying a compound which modulates the activity of a G protein-coupled receptor, comprising:

- 5 a) isolating and mutating a nucleic acid molecule encoding a wild-type G protein-coupled receptor, wherein mutating the nucleic acid molecule comprises creating at least one nucleotide mutation resulting in an amino acid change in the wild-type G protein-coupled receptor, such amino acid being expressed in transmembrane domain III or VI, thereby producing a second
- 10 nucleic acid molecule sequence encoding a mutant receptor;
- b) separately assaying the basal activity of the wild-type receptor, the mutant receptor and a negative control;
- c) determining whether the activity of the mutant receptor is greater than the wild-type receptor and the negative control; and if the basal activity of the
- 15 mutant receptor is greater, then
- d) assaying the compound at the mutant receptor to determine whether the compound is an agonist, inverse agonist, partial agonist, or antagonist of the G protein-coupled receptor;
- wherein the wild-type G protein-coupled receptor is a human GPR88 receptor, or a
- 20 variant thereof.

2. The method of claim 1, wherein the wild-type G protein-coupled receptor comprises SEQ ID NO: 2.

25 3. The method of claim 1, wherein the amino acid change in transmembrane domain III of the wild-type G protein-coupled receptor comprises an amino acid change of asparagine (N) to alanine (A).

30 4. The method of claim 1, wherein the amino acid change in transmembrane domain VI of the wild-type G protein-coupled receptor comprises an amino acid change selected from

the group consisting of glycine (G) to histidine (H), glycine (G) to glutamic acid (E), glycine (G) to lysine (K), leucine (L) to histidine (H), leucine (L) to glutamic acid (E), leucine (L) to lysine (K), leucine (L) to phenylalanine (F), leucine (L) to glutamic acid (E), phenylalanine (F) to valine (V), phenylalanine (F) to leucine (L), and phenylalanine (F) to alanine (A).

- 5 5. The method of claim 1, wherein the human GPR88 receptor variant comprises a further mutation which does not affect the activity of the receptor.
- 10 6. The method of claim 2, wherein the amino acid change in transmembrane domain III of the wild-type G protein-coupled receptor comprises an amino acid change asparagine (N) to alanine (A) at or around amino acid position 137 of SEQ ID NO: 2.
- 15 7. The method of claim 2, wherein the amino acid change in transmembrane domain VI of the wild-type G protein-coupled receptor comprises an amino acid change selected from the group consisting of glycine (G) to histidine (H) at or around amino acid position 283, glycine (G) to glutamic acid (E) at or around amino acid position 283, glycine (G) to lysine (K) at or around amino acid position 283, leucine (L) to histidine (H) at or around amino acid position 284, leucine (L) to glutamic acid (E) at or around amino acid position 284, leucine (L) to lysine (K) at or around amino acid position 284, leucine (L) to phenylalanine (F) at or around amino acid position 289, leucine (L) to glutamic acid (E) at or around amino acid position 289, phenylalanine (F) to valine (V) at or around amino acid position 293, phenylalanine (F) to leucine (L) at or around amino acid position 293, and phenylalanine (F) to alanine (A) at or around amino acid position 293 of SEQ ID NO: 2.
- 20
- 25

8. The method of claim 1, wherein the nucleic acid molecule encoding a mutant receptor comprises a nucleic acid molecule having a nucleotide sequence selected from the group consisting of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26.
- 5 9. The method of claim 1, wherein the mutant receptor comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27.
- 10 10. The method of claim 1, further comprising determining whether the compound is a neutral antagonist, in the presence of an agonist.
11. The method of any one of the preceding claims, wherein the wild-type and mutated nucleic acid molecules comprise cDNA.
- 15 12. The method of claim 1, wherein assaying the basal activity of the receptor is done in the absence of a receptor ligand.
13. The method of claim 1, wherein assaying the basal activity of the receptors is done in the presence of a receptor agonist or antagonist.
- 20 14. The method of claim 1, wherein assaying the basal activity of the receptors comprises transfecting a first host cell with a nucleic acid molecule encoding a wild-type receptor, and a second host cell with a nucleic acid molecule encoding a mutant receptor.
- 25 15. The method of claim 14, wherein the first and second host cells are cotransfected with a reporter construct comprising a promoter operably linked to the receptor wild-type receptor or the mutant receptor.
16. The method of claim 15, wherein the promoter comprises a response element.
- 30

17. The method of claim 16, wherein the response element is sensitive to a signal induced by the receptor.

18. The method of any one of claims 14-17, wherein the receptor induces an signal via a G protein selected from $G\alpha_q$, $G\alpha_i$, or $G\alpha_s$.

19. The method of claim 18, wherein the G protein is $G\alpha_q5i$.

20. The method of claim 16, wherein the response element is selected from a somatostatin promoter, serum response element, cAMP response element, NF κ B response element, NFAT response element, and AP-1 response element.

21. The method of any one of claims 15-20, wherein the reporter construct produces a reporter molecule.

22. The method of claim 21, wherein the reporter molecule is a transcriptional reporter molecule.

23. The method of claim 22, wherein the reporter molecule is luciferase, beta-galactosidase, or chloramphenicol acetyl transferase.

24. The method of claim 1, wherein the assay is a transcriptional reporter assay.

25. The method of claim 24, wherein the assay is a transcriptional reporter assay selected from a cAMP, luciferase reporter, beta-galactosidase reporter, chloramphenicol acetyl transferase reporter, intracellular calcium measurement, mitogenesis, GTP γ S, MAP kinase activity, inositol phosphate, arachidonic acid release, or extracellular acidification rate assay.

26. The method of claim 1, wherein the basal activity of the mutant receptor is at least 25% greater than the basal activity of the wild-type receptor.

27. The method of claim 1, wherein the nucleic acid molecule encoding the mutant receptor
5 comprises at least 10, 15, 20 or 25 consecutive nucleotides of SEQ ID NO: 3.

28. The method of claim 1, wherein the nucleic acid molecule encoding the wild-type receptor comprises at least 10, 15, 20 or 25 consecutive nucleic acids of SEQ ID NO: 1.

10 29. A method for detecting or measuring G protein-coupled receptor activity comprising:

a) cotransfecting a host cell with an expression vector comprising a promoter operably linked to a nucleic acid molecule encoding a constitutively active mutant G protein-coupled receptor, wherein the receptor can induce a ligand-independent intracellular signal, and a reporter construct comprising a response element operably linked to a reporter gene,
15 wherein the reporter gene is a modified reporter gene that is rapidly expressed by the intracellular signal induced by the receptor, and

b) detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof following the cotransfection of the expression vector and reporter construct.

20 30. The method of claim 29, wherein the amount of the expressed reporter gene is detectable by use of an assay that directly or indirectly measures the activity of the polypeptide encoded by the expressed reporter gene.

31. The method of claim 29, wherein detecting or measuring the amount of the expressed
25 reporter gene in the cell or a lysate thereof is by means of measuring the level of activity that the expressed reporter gene has on an enzyme substrate.

32. The method of claim 29, wherein detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof is done within 4 hours.

33. The method of claim 29, wherein detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof is done within 5 hours.

34. The method of claim 29, wherein detecting or measuring the amount of the expressed reporter gene in the cell or a lysate thereof is done within 6 hours.

35. The method of claim 29, wherein the response element is selected from a somatostatin promoter, serum response element, cAMP response element, NFκB response element, NFAT response element, and AP-1 response element.

36. The method of claim 29, wherein the reporter construct comprises a luciferase reporter gene, beta-galactosidase reporter gene, or chloramphenicol acetyl transferase reporter gene.

37. The method of any one of claims 29-36, further comprising cotransfecting the host cell with a chimeric G protein.

38. The method of any one of claims 29-36, further comprising cotransfecting the host cell with a Gαq5i.

39. The method of any one of claims 29-36, wherein the reporter construct comprises SEQ ID NO: 28.

40. The method of any one of claims 29-36, wherein the reporter construct encodes a polypeptide having the amino acid sequence of SEQ ID NO: 29.

41. The method of any one of claims 29-36, wherein the expressed reporter gene is a luciferase polypeptide comprising SEQ ID NO: 29.

42. The method of claim 29, wherein the reporter gene is a modified reporter gene comprising SEQ ID NO: 28.

43. The method of any one of claims 29-42, wherein the host cell is contacted with a test compound or test ligand.

44. The method of claim 43, wherein an increased amount of expressed reporter gene is detected in the host cell contacted with a test compound or test ligand compared to a host cell that is not contacted with a test compound or test ligand.

45. The method of claim 43, wherein a decreased amount of expressed reporter gene is detected in the host cell contacted with a test compound or test ligand compared to a host cell that is not contacted with a test compound or test ligand.

46. The method of claim 29, wherein the nucleic acid molecule comprises SEQ ID NO: 3.

47. The method of claim 29, wherein the constitutively active mutant G protein-coupled receptor comprises a polypeptide encoded by SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26.

48. The method of claim 29, wherein the constitutively active mutant G protein-coupled receptor comprises a polypeptide having the amino acid sequence of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 or 27.

49. An isolated nucleic acid molecule comprising SEQ ID NO: 3 which encodes for a human GPR88 receptor with unaltered expression in a host cell.

50. An isolated nucleic acid molecule comprising SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26.

51. A cell transfected with the isolated nucleic acid molecule of claim 50.

52. A vector comprising the isolated nucleic acid molecule of claim 50.

5 53. An isolated nucleic acid molecule which is complementary to the nucleic acid of claim 49.

54. An isolated nucleic acid molecule encoding a polypeptide comprising an amino acid
sequence selected from the group consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19,
10 21, 23, 25 and 27, or variants thereof.

55. An isolated polypeptide having an amino acid sequence selected from the group
consisting of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27, or variants
thereof.

15 56. A fragment of a polypeptide of claim 55, wherein the fragment comprises an amino acid
sequence having at least 293 contiguous amino acids.

57. A fragment of a polypeptide of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, or 25,
20 wherein the fragment comprises an amino acid sequence having at least 50 contiguous
amino acids comprising transmembrane domain VI.

58. A fragment of a polypeptide of SEQ ID NO: 27, wherein the fragment comprises an
amino acid sequence having at least 50 contiguous amino acids comprising
25 transmembrane domain III.

59. An isolated nucleic acid encoding the polypeptide of claim 56, 57 or 58.

1/8

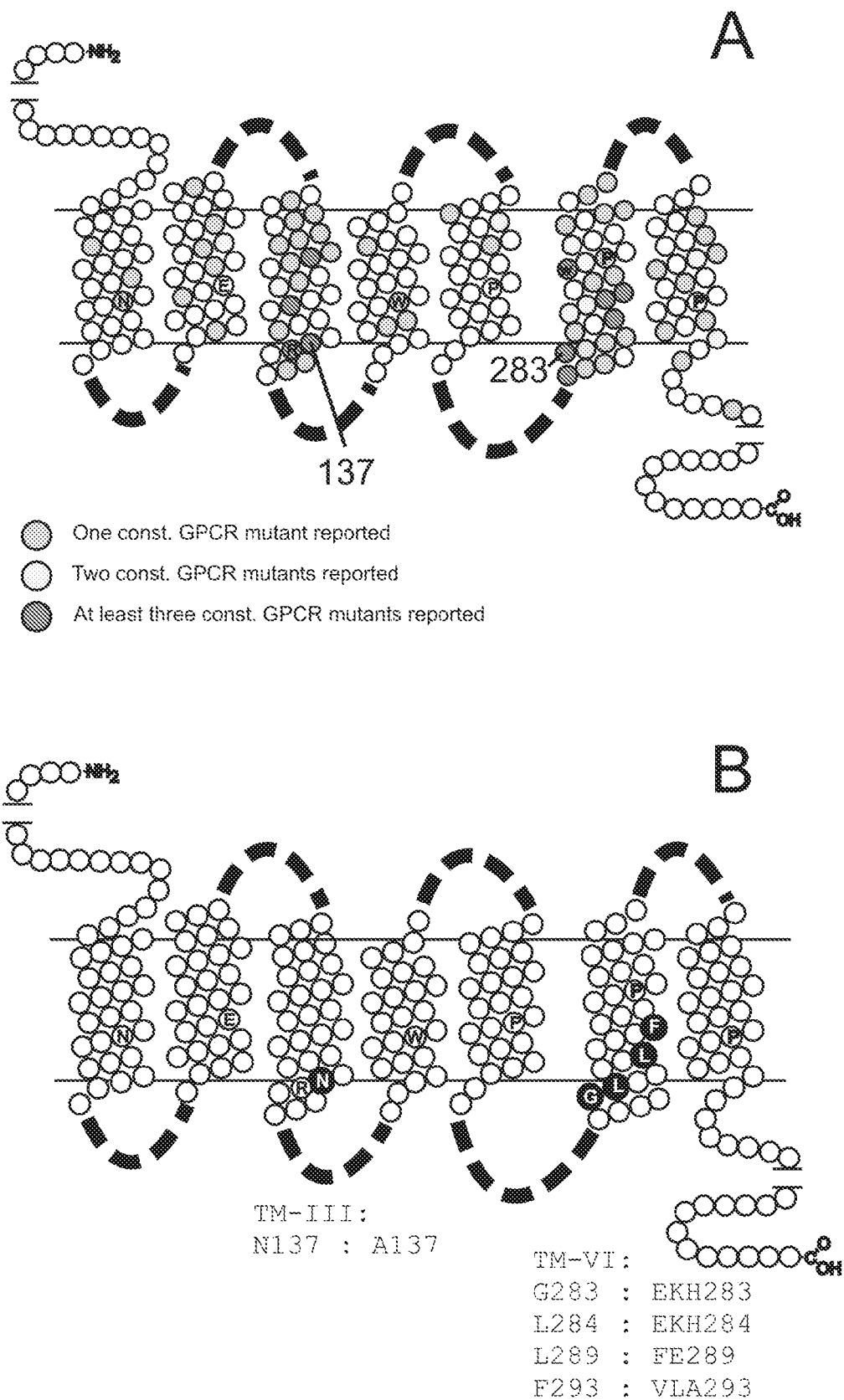


Figure 1.

2/8

MTNSSSTSTSSSTTGGSLLLLCEEEESWAGRRIPVSLLYSGLAIGGTLANGM
TMI

VIYLVSSFRKLQTTSNAFIVNGCAADLSVCALWMPQEAVLGLLPTGSAEPP
TMII

ADWDGAGGSYRLLRGGLLGLGLTVSLLSHCLVAL**N**RYLLITRAPATYQALY
TMIII

QRRHTAGMLALSWALALGLVLLLPPWAPRPGAAPPRIHYPALLAAAALLAQ
TMIV TMV

TALLLHCYLGIVRRVRVSVKRVSVLNFHLLHQLPGCAAAAAAFPGAQHAPG

PGGAHHPAQQAQPLPPALHPRRAQRRLSGLSVLL**L**CCV**F**LLATQPLVWVSLA
TMVI

SGFSLPVPWGVQAASWLLCCALSALNPLLYTWRNEEFRRSVRSVLPGVGDA
TMVII

AAAAVAATAVPAVSQAQLGTRAAGQHW

Figure 2.

3/8

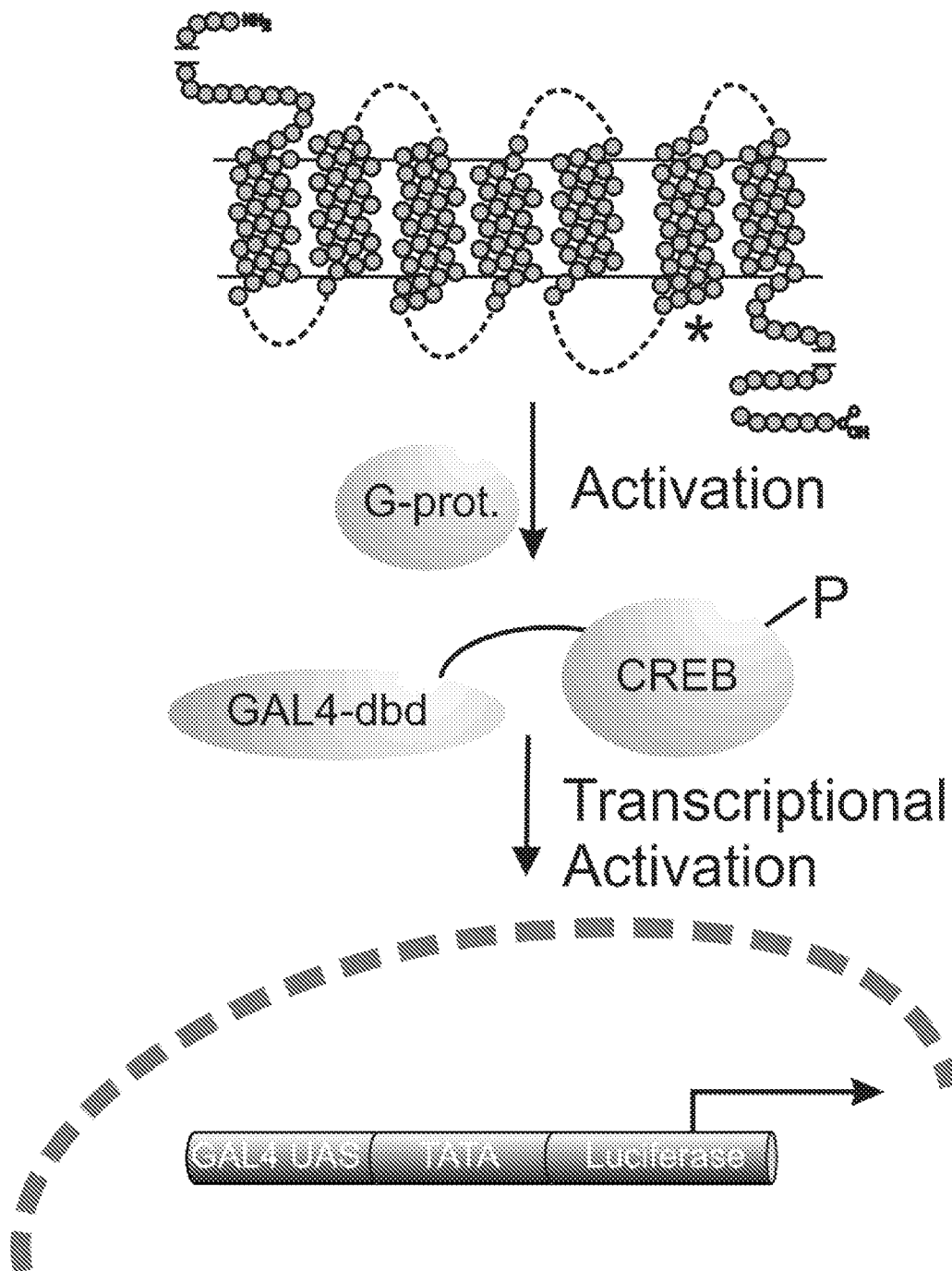
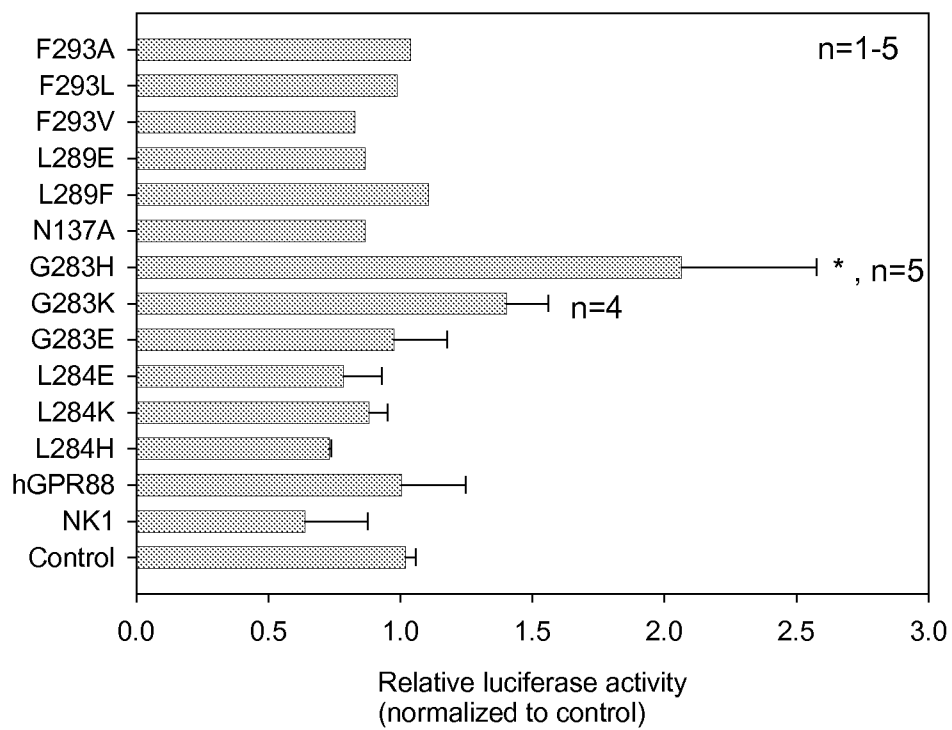


Figure 3.

4/8

**Figure 4.**

5/8

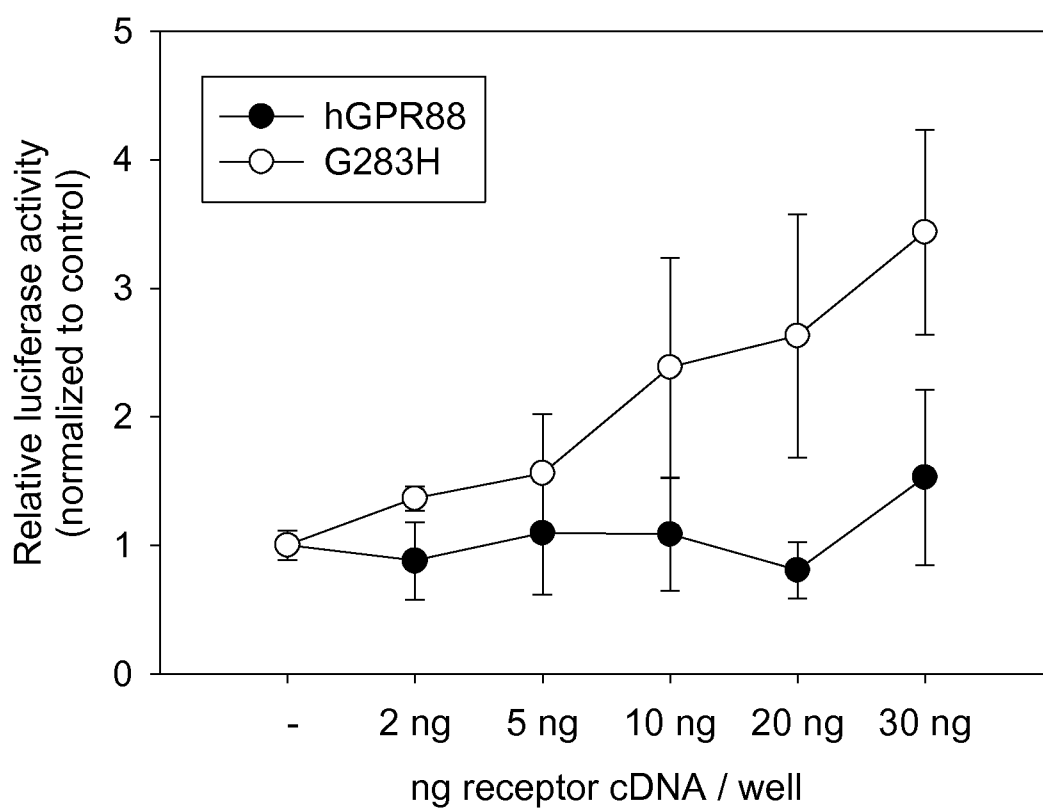
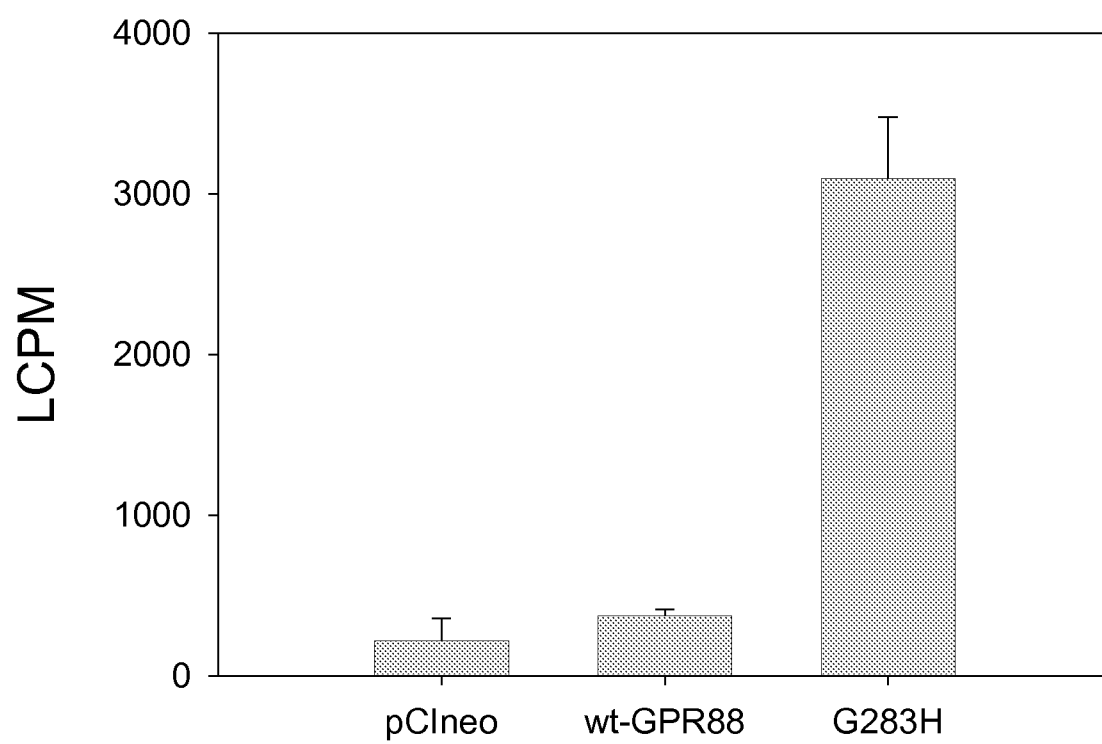


Figure 5.

6/8**Figure 6.**

7/8

KT-001



Figure 7.

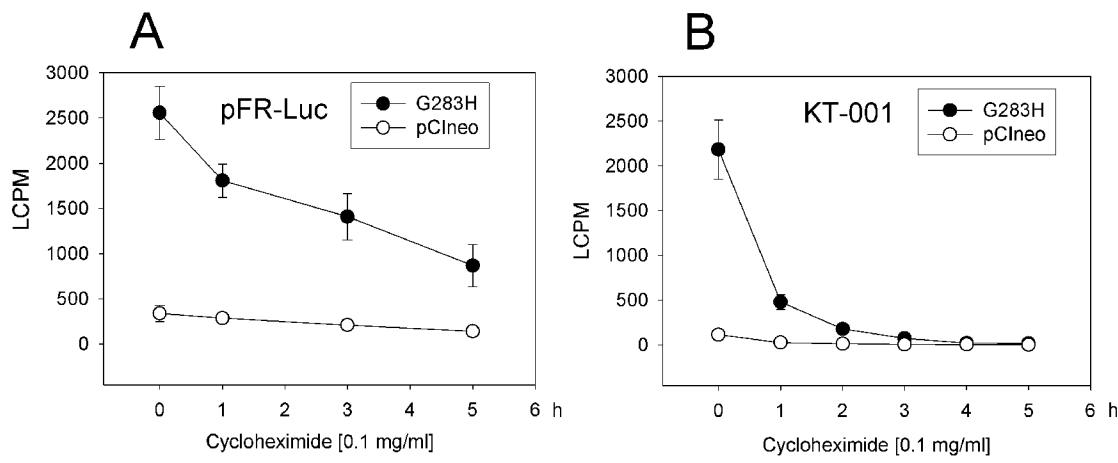


Figure 8.