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(71) Applicant *(for all designated States except US)*: **HEPTARES THERAPEUTICS LIMITED** [GB/GB]; BioPark, Broadwater Road, Welwyn Garden City, Hertfordshire AL7 3AX (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LEBON, Guillaume Pierre** [FR/GB]; MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (GB). **TATE, Christopher Gordon** [GB/GB]; MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (GB). **JAZAYERI-DEZFULY, Seyed Ali** [GB/GB]; Heptares Therapeutics Ltd, BioPark, Broadwater Road, Welwyn Garden City, Hertfordshire AL7 3AX (GB).

(74) Agent: **THOMAS, Philip John Duval**; Park View House, 58 The Ropewalk, Nottingham, Nottinghamshire NG1 5DD (GB).

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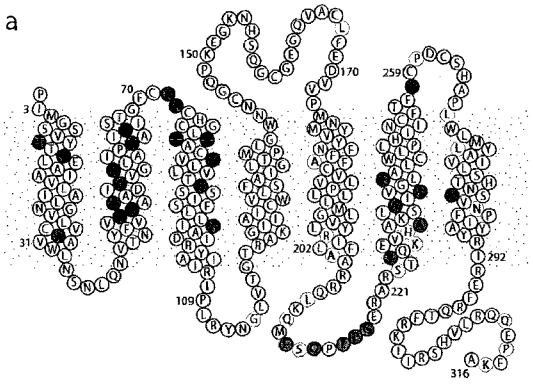
(54) Title: MUTANT PROTEINS AND METHODS FOR PRODUCING THEM

FIGURE 1 (Page 1 of 2)



Thermostabilising mutations: I10, V12, W29, S47, L48, A50, A54, V57, F62, T65, A72, A73, F79, I80, F83, Q89, A97, E212, Q214, L216, P217, G218, L225, A231, A236, I238, F242, F258, V282
Mutations selected with unliganded receptor: G114, L167, A184, R199, A203, L208, Q210, S213, S223, K227, H230, P260, L267, L272, N284, Q311, P313, K315
Mutations selected with both assays: S90, G118, E219, R220, T224, Q226, L241, S263, T279

(57) Abstract: A mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of lie 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, lie 80, Phe 83, Gin 89, Ala 97, Glu 212, Gin 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, lie 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

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MUTANT PROTEINS AND METHODS FOR PRODUCING THEM

The present invention relates to mutant 7-transmembrane spanning receptors (7-TMRs) or G protein coupled receptors (GPCRs) with increased stability, and methods for producing them. Such proteins are more likely to be crystallisable, and hence amenable to structure determination, than the parent proteins. They are also useful for drug discovery and development studies.

Over the past 20 years the rate of determination of membrane protein structures has gradually increased, but most success has been in crystallising membrane proteins from bacteria rather than from eukaryotes. Bacterial membrane proteins have been easier to overexpress using standard techniques in *Escherichia coli* than eukaryotic membrane proteins and the bacterial proteins are sometimes far more stable in detergent, detergent-stability being an essential prerequisite to purification and crystallisation. Genome sequencing projects have also allowed the cloning and expression of many homologues of a specific transporter or ion channel, which also greatly improves the chances of success during crystallisation. Although the structures of over 100 unique polytopic integral membrane proteins have been determined (see http://blanco.biomol.uci.edu/), less than 10% of these membrane proteins are of mammalian origin and over half were purified from natural sources and are stable in detergent solutions. Apart from the difficulties in overexpressing eukaryotic membrane proteins, they often have poor stability in detergent solutions, which severely restricts the range of crystallisation conditions that can be explored without their immediate denaturation or precipitation. Ideally, membrane proteins should be stable for many days in any given detergent solution, but the detergents that are best suited to growing diffraction-quality crystals tend to be the most destabilising detergents *ie* those with short aliphatic chains and small or charged head groups. It is also the structures of human membrane proteins that we would like to solve, because these are required to help the development of therapeutic agents by the pharmaceutical industry; often there are substantial differences in the pharmacology of receptors, channels and transporters from different mammals, whilst yeast and bacterial genomes may not include any homologous proteins. There is thus an overwhelming need to develop a generic strategy that will allow the production of detergent-stable eukaryotic integral membrane proteins for crystallisation and structure determination and potentially for other purposes such as drug screening, bioassay and biosensor applications.

Membrane proteins have evolved to be sufficiently stable in the membrane to ensure cell viability, but they have not evolved to be stable in detergent solution, suggesting that membrane proteins could be artificially evolved and detergent-stable mutants isolated. This was subsequently demonstrated for two bacterial proteins, diacylglycerol kinase (DGK) and bacteriorhodopsin. Random mutagenesis of DGK identified specific point mutations that increased thermostability and, when combined, the effect was additive so that the optimally stable mutant had a half-life of 35 minutes at 80°C compared with a half-life of 6 minutes at 55°C for the native protein. It was shown that the trimer of the detergent-resistant DGK mutant had become stable in SDS and it is thus likely that stabilisation of the oligomeric state played a significant role in thermostabilisation. Although the aim of the mutagenesis was to produce a membrane protein suitable for crystallisation, the structure of DGK has yet to be determined and there have been no reports of successful crystallization. A further study on bacteriorhodopsin by cysteine-scanning mutagenesis along helix B demonstrated that it was not possible to predict which amino acid residues would lead to thermostability upon mutation nor, when studied in the context of the structure, was it clear why thermostabilisation had occurred.

GPCRs constitute a very large family of proteins that control many physiological processes and are the targets of many effective drugs. Thus, they are of considerable pharmacological importance. A list of GPCRs is given in Foord *et al* (2005) *Pharmacol Rev.* **57**, 279-288, which is incorporated herein by reference. GPCRs are generally unstable when isolated, and until recently, it has not been possible to crystallise any except bovine rhodopsin, which is exceptionally stable in its native unilluminated state.

By GPCRs we include all 7-TMRs within the GPCR superfamily, including receptors that signal to G proteins as well as those receptors which do not signal to G proteins.

GPCRs are druggable targets and reference is made to Overington *et al* (2006) *Nature Rev. Drug Discovery* **5**, 993-996 which indicates that more than a quarter of current drugs target GPCRs. There are 52 GPCR targets for orally available drugs out of a total of 186 total targets in this category. GPCRs are thought to exist in multiple distinct conformations which are associated with different pharmacological classes of ligand such as agonists and antagonists, and to cycle between these conformations in order to function (*Kenakin T.* (1997) *Ann N Y Acad Sci* **812**, 116-125).

The inventors have previously developed various methodologies for selecting mutations that improve the stability of GPCRs, and, in addition, that preferentially lock the receptor in a specific biologically relevant conformation. Such methods are described in WO 2008/114020 and in WO 2009/071914, incorporated herein by reference.

As described in Example 1, the inventors have now identified further mutant GPCRs with increased stability relative to a parent GPCR. Specifically, they have demonstrated that by mutating any of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 in the human adenosine A_{2A} receptor, the stability of the GPCR can be increased. Further, the inventors have demonstrated that by combining these stabilising mutations with further stabilising mutations known in the art, stability may be further increased. These mutant GPCRs and methods for producing them are described below.

Accordingly, a first aspect of the invention provides a mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

The invention also provides a mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid (i) at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

A second aspect of the invention provides a method of producing a mutant GPCR with increased stability relative to its parent GPCR, the method comprising making one or

more mutations in the amino acid sequence that defines a parent GPCR at a position corresponding to one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

The invention also provides a method of producing a mutant GPCR with increased stability relative to its parent GPCR, the method comprising making one or more mutations in the amino acid sequence that defines a parent GPCR (i) at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

It will be understood that the methods of the second aspect of the invention may be carried out to produce any mutant GPCR described herein.

Preferably, the mutant GPCR of the invention, and the mutant GPCR produced by the method of the invention, has, compared to the corresponding parent receptor, a different amino acid at a position which corresponds to any one or more of Val 12, Ser 47, Ala 50, Phe 62, Thr 65, Phe 79, Phe 83, Gln 89, Ala 236, Ile 238, Phe 242, and Val 282, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

In another preferred embodiment, the mutant GPCR of the invention, and the mutant GPCR produced by the method of the invention, has, compared to the corresponding parent receptor, (i) a different amino acid at a position which corresponds to any one or more of Val 12, Phe 62, Thr 65, Phe 79, Phe 83, Gln 89, Ala 236, Ile 238, Phe 242, and Val 282, and (ii) a different amino acid at a position which corresponds to Leu 48, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

In yet another preferred embodiment, the mutant GPCR of the invention, and the mutant GPCR produced by the method of the invention, has, compared to the corresponding parent receptor, (i) a different amino acid at a position which corresponds to any one or more of Val 12, Ala 54, Phe 62, Thr 65, and Phe 79, (ii) a different amino acid at a position which corresponds to Leu 48, and (iii) a different amino acid at a position which corresponds to Gln 89, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

In yet another preferred embodiment, the mutant GPCR of the invention, and the mutant GPCR produced by the method of the invention, has, compared to the corresponding parent receptor, (i) a different amino acid at a position which corresponds to any one or more of Ala 50, Ala 54, Phe 83 and Ser 263, (ii) a different amino acid at a position which corresponds to Leu 48, (iii) a different amino acid at a position which corresponds to Gln 89, and (iii) a different amino acid at a position which corresponds to Thr 65, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

By "corresponding amino acid residue" we include the meaning of the amino acid residue in another GPCR (eg adenosine receptor) which aligns to the given amino acid residue in human adenosine A_{2A} receptor when the human adenosine A_{2A} receptor and the other GPCR are compared using MacVector and CLUSTALW, as described further below.

Suitable GPCRs for use in the practice of the invention include, but are not limited to adenosine receptor, in particular adenosine A_{2A} receptor (gene name: ADORA2A), muscarinic receptor, serotonin receptor (eg $5HT_{2C}$; gene name HTR2C), β-adrenergic receptor (e.g. βAR-1; gene name: ADRB1), neurotensin receptor (NTS_1; gene name: NTSR1), and orexin receptor (e.g. OX_2; gene name: HTR2C). In addition, the International Union of Pharmacology produces a list of GPCRs (Foord *et al* (2005) *Pharmacol. Rev.* **57**, 279-288, incorporated herein by reference and this list is periodically updated at http://www.iuphar-db.org/GPCR/ReceptorFamiliesForward). Thus, the GPCR may be any of a mutant adenosine receptor, a mutant β-adrenergic receptor, a mutant neurotensin receptor, a mutant muscarinic acid receptor, a mutant 5-hydroxytryptamine receptor, a mutant adrenoceptor, a mutant anaphylatoxin receptor, a mutant angiotensin receptor, a mutant apelin receptor, a mutant bombesin receptor, a

mutant bradykinin receptor, a mutant cannabinoid receptor, a mutant chemokine receptor, a mutant cholecystokinin receptor, a mutant dopamine receptor, a mutant endothelin receptor a mutant free fatty acid receptor, a mutant bile acid receptor, a mutant galanin receptor, a mutant motilin receptor, a mutant ghrelin receptor, a mutant glycoprotein hormone receptor, a mutant GnRH receptor, a mutant histamine receptor, a mutant KiSS1-derived peptide receptor, a mutant leukotriene and lipoxin receptor, a mutant lysophospholipid receptor, a mutant melanin-concentrating hormone receptor, a mutant melanocortin receptor, a mutant melatonin receptor, a mutant neuromedin U receptor, a mutant neuropeptide receptor, a mutant *N*-formylpeptide family receptor, a mutant nicotinic acid receptor, a mutant opiod receptor, a mutant opsin-like receptor, a mutant orexin receptor, a mutant P2Y receptor, a mutant peptide P518 receptor, a mutant platelet-activating factor receptor, a mutant prokineticin receptor, a mutant prolactin-releasing peptide receptor, a mutant prostanoid receptor, a mutant protease-activated receptor, a mutant relaxin receptor, a mutant somatostatin receptor, a mutant SPC/LPC receptor, a mutant tachykinin receptor, a mutant trace amino receptor, a mutant thryotropin-releasing hormone receptor, a mutant urotensin receptor, a mutant vasopressin/oxytocin receptor, a mutant orphan GPCR, a mutant calcitonin receptor, a mutant corticotropin releasing factor receptor, a mutant glucagon receptor, a mutant parathyroid receptor, a mutant VIP/PACAP receptor, a mutant LNB7TM receptor, a mutant GABA receptor, a mutant metabotropic glutamate receptor, and a mutant calcium sensor receptor (see Table 1 of Foord *et al* (2005) *Pharmacol. Rev.* **57**, 279-288, incorporated herein by reference).

It will be noted that GPCRs are divided into different classes, principally based on their amino acid sequence similarities, for example Classes 1, 2 and 3 whose archetypes are rhodopsin, the secretin receptor and the metabotropic glutamate receptor 1. GPCRs are also divided into families by reference to the natural ligands to which they bind. All GPCRs, including 7-TMRs in the superfamily of GPCRs, are included in the scope of the invention.

Preferably, the mutant GPCR is a mutant adenosine receptor, or a mutant serotonin receptor, or a mutant β-adrenergic receptor, or a mutant neurotensin receptor, or a mutant muscarinic receptor, or a mutant orexin receptor.

The amino acid sequences (and the nucleotide sequences of the cDNAs which encode them) of many GPCRs are readily available, for example by reference to GenBank. In

particular, Foord *et al supra* gives the human gene symbols and human, mouse and rat gene IDs from Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez). It should be noted, also, that because the sequence of the human genome is substantially complete, the amino acid sequences of human GPCRs can be deduced therefrom. Figure 8 lists the amino acid sequences of various Class 1 GPCRs.

Although the parent GPCR may be any GPCR, it is particularly preferred if it is a eukaryotic GPCR, that is the cDNA or gene encoding the GPCR is a eukaryotic cDNA or gene. For example, it is particularly preferred if the parent GPCR is a vertebrate GPCR such as a GPCR from a mammal. It is particularly preferred if the parent GPCR is from rat, mouse, rabbit or dog or non-human primate or human.

It is appreciated that the amino acid sequence defining the parent GPCR need not be an amino acid sequence defining the naturally occurring protein. Conveniently, it may define an engineered version which is capable of expression in a suitable host organism, such as in bacteria, yeast, insect cells or in mammalian cells. The amino acid sequence defining the parent GPCR may be an amino acid sequence defining a truncated form of the naturally occurring protein (truncated at either or both ends), or an amino acid sequence defining a fusion, either to the naturally occurring protein or to a fragment thereof, or an amino acid sequence that contains mutations compared to the naturally-occurring sequence. Alternatively or additionally, the amino acid sequence defining the parent GPCR, compared to a naturally-occurring GPCR, may be modified in order to improve, for example, solubility or proteolytic stability (eg by truncation, deletion of loops, mutation of glycosylation sites or mutation of reactive amino acid side chains such as cysteine). In any event, it will be appreciated that the amino acid sequence defining the parent GPCR is one that defines a GPCR that is able to bind to a ligand. The ligand may bind to the naturally occurring GPCR, or to a mutant thereof, or to a derivative of the naturally occurring GPCR or mutant thereof. By 'derivative' we include the meaning of a GPCR which compared to the naturally occurring GPCR has been chemically modified, for example by attachment of any chemical moiety to one or more amino acid side chains, or by the insertion of any chemical moiety within the amino acid sequence, but which derivative retains the ability to bind to a ligand.

Conveniently, the amino acid sequence defining a parent GPCR is one that defines a GPCR which, on addition of an appropriate ligand, can affect any one or more of the downstream activities which are commonly known to be affected by activation of G

proteins, or other pathways independent of G proteins such as those which include arrestins. For example, where the parent GPCR is a 7-TMR that can signal independently of a G protein (e.g. smoothened or a mutant GPCR which has lost G protein signalling ability but retains signalling to other pathways), the amino acid sequence may be one that defines a parent GPCR which, on addition of an appropriate ligand, activates a G protein independent signalling pathway.

It is particularly preferred if the mutant GPCR is one that has at least 20% amino acid sequence identity when compared to the given human adenosine A_{2A} receptor whose sequence is set out in Figure 7, as determined using MacVector and CLUSTALW (Thompson *et al* (1994) *Nucl. Acids Res.* **22**, 4673-4680). More preferably, the mutant GPCR has at least 30% or at least 40% or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90% amino acid sequence identity, such as at least 95% or 99% amino acid sequence identity. There is generally a higher degree of sequence conservation at the adenosine binding site.

Any suitable method may be used to align two polypeptide sequences, including but not limited to those described in Computational Molecular Biology (A. M. Lesk, ed., Oxford University Press 1988); Biocomputing: Informatics and Genome Projects (D. W. Smith, ed., Academic Press 1993); Computer Analysis of Sequence Data (Part 1, A. M. Griffin and H. G. Griffin, eds., Humana Press 1994); G. von Heinje, Sequence Analysis in Molecular Biology (Academic Press 1987); Sequence Analysis Primer (M. Gribskov and J. Devereux, eds., M. Stockton Press 1991); and Carillo et al., SIAMJ. Applied Math. 48: 1073 (1988). Preferred methods to align polypeptides are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs.

Preferred computer program methods to align polypeptide sequences and to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux et al., Nuc. Acids Res. 12: 387 (1984); Genetics Computer Group, University of Wisconsin, Madison, WI), BLASTP, BLASTN, and FASTA (Atschul et al., J. Mol. Biol. 215: 403-10 (1990)). The BLAST X program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (Altschul et al., BLAST Manual (NCB NLM NIH, Bethesda, MD); Altschul et al., 1990, supra). The well-known Smith Waterman algorithm may also be used to determine identity.

By way of example, using the computer algorithm GAP (Genetics Computer Group), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span," as determined by the algorithm). A gap opening penalty (which is calculated as 3X the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 0.1X the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., 5 Atlas of Protein Sequence and Structure (Supp. 3 1978) for the PAM250 comparison matrix; see Henikoff et al., Proc. Natl. Acad. Sci USA 89: 10915-19 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm. Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48: 443-53 (1970). Comparison matrix: BLOSUM 62 from Henikoff et al., Proc. Natl. Acad. Sci. U. S. A. 89: 10915-19 (1992) Gap Penalty: 12 Gap Length Penalty: 4 Threshold of Similarity: 0 The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

Changes to a single amino acid within the GPCR may increase the stability of the protein compared to the parent protein. Thus, in one embodiment, the mutant GPCR of the invention contains a single changed amino acid compared to the parent protein.

However, it is appreciated that a further increase in stability may be obtained by changing more than one of the amino acids of the parent protein. Thus, the mutant GPCRs of the invention may contain two or more changed amino acids compared to the parent protein. Typically, the mutant GPCR contains, compared to the parent protein, from 1 to 11 changed amino acids, preferably from 1 to 10 or from 1 to 9 or from 1 to 8 or from 1 to 7 or from 1 to 6, such as 2 to 6, for example 2, 3, 4, 5 or 6 changed amino acids. However, it is appreciated that the total number of mutations to confer increased stability may be more than this, and will ultimately vary from receptor to receptor, depending on various factors such as the intrinsic stability of the parent receptor.

In a preferred embodiment, the mutant GPCR of the invention is one that comprises two stabilising mutations. Thus, the mutant GPCR may be one which when compared to the corresponding parent receptor has a different amino acid at the positions that correspond to Leu 48 and Val 12; or to L48 and Phe 62; or to Leu 48 and Thr 65; or to Leu 48 and Phe 79; or to Leu 48 and Phe 83; or to Leu 48 and Gln 89; or to Leu 48 and Ala 236; or to Leu 48 and Ile 238; or to Leu 48 and Phe 242; or to Leu 48 and Val 282, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

In another preferred embodiment, the mutant GPCR of the invention is one that comprises three stabilising mutations. Thus, the mutant GPCR may be one which when compared to the corresponding parent receptor has a different amino acid at the positions that correspond to Leu 48 and Gln 89 and Val 12; or to L48 and Gln 89 and Ala 54; or to Leu 48 and Gln 89 and Phe 62; or to Leu 48 and Gln 89 and Phe 79; or to Leu 48 and Gln 89 and Thr 65, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

In another preferred embodiment, the mutant GPCR of the invention is one that comprises four stabilising mutations. Thus, the mutant GPCR may be one which when compared to the corresponding parent receptor has a different amino acid at the positions that correspond to Leu 48 and Gln 89 and Thr 65 and Ala 50; or to L48 and Gln 89 and Thr 65 and Ala 50; or to Leu 48 and Gln 89 and Thr 65 and Phe 83; or to Leu 48 and Gln 89 and Thr 65 and Ser 263, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

Mutations can be made in an amino acid sequence defining a parent GPCR using any suitable technique known in the art. For example, conventional site-directed mutagenesis may be employed, or polymerase chain reaction-based procedures may be used, such that particular amino acid residues are independently replaced with other amino acid residues. Molecular biological methods for cloning and engineering genes and cDNAs, for mutating DNA, and for expressing polypeptides from polynucleotides in host cells are well known in the art, as exemplified in "Molecular cloning, a laboratory manual", third edition, Sambrook, J. & Russell, D.W. (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Typically, making one or more mutations in the amino acid sequence that defines a parent GPCR comprises replacing one or more amino acids by Ala, although it may be replaced by any other amino acid. For example, if a particular amino acid within any of the windows described above is Ala, it may conveniently be replaced by Leu. Alternatively, the amino acid may be replaced by Gly for example, which may allow a closer packing of neighbouring helices that may lock the protein in a particular conformation. If the amino acid is Gly, it may conveniently be replaced by Ala for example.

Although the amino acid used to replace a given amino acid at a particular position is typically a naturally occurring amino acid, typically an "encodeable" amino acid, it may be a non-natural amino acid (in which case the protein is typically made by chemical synthesis or by use of non-natural amino-acyl tRNAs). An "encodeable" amino acid is one which is incorporated into a polypeptide by translation of mRNA. It is also possible to create non-natural amino acids or introduce non-peptide linkages at a given position by covalent chemical modification, for example by post-translational treatment of the protein or semisynthesis. These post-translational modifications may be natural, such as phosphorylation, glycosylation or palmitoylation, or synthetic or biosynthetic.

Mutant adenosine receptor

In a particularly preferred embodiment, the mutant GPCR is a mutant adenosine receptor. Adenosine receptors are well known in the art. They share sequence homology to each other and bind to adenosine. The invention provides particular mutant adenosine receptors and methods for producing them.

In one embodiment, the mutant GPCR with increased stability relative to its parent GPCR is a mutant adenosine receptor which, when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

In another embodiment, the mutant GPCR with increased stability relative to its parent GPCR is a mutant adenosine receptor which when compared to the corresponding

parent receptor, has a different amino acid (i) at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

Other preferences for mutant adenosine receptors include those can contain two, three or four stabilising mutations as described above. Thus, the mutant GPCR may be a mutant adenosine receptor (e.g. A_{2A} receptor) in which, compared to its parent, has a different amino acid at positions that correspond to Leu 48 and Gln 89; or to Leu 48 and Gln 89 and Thr 65; or to Leu 48 and Gln 89 and Thr 65 and Ala 54, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

The mutant adenosine receptor may be a mutant of any adenosine receptor provided that it is mutated at a position which corresponds to any one or more of the above mentioned residues according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

As is described in Example 1 below, replacement of each of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, leads to an increase in thermostability when measured with the agonist ^{3}H-NECA (see Table 1).

Thus, the mutant GPCR may be a mutant human adenosine A_{2A} receptor in which, compared to its parent, one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 have been replaced by another amino acid residue.

Similarly, the mutant GPCR may be a mutant human adenosine A_{2A} receptor in which, compared to its parent, one or more of (i) at a position which corresponds to any one or

more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315 have been replaced by another amino acid residue.

The mutant GPCR may also be a mutant adenosine receptor from another source in which one or more corresponding amino acids in the parent receptor are replaced by another amino acid residue. For the avoidance of doubt, the parent may be an adenosine receptor which has a naturally-occurring sequence, or it may be a truncated form or it may be a fusion, either to the naturally-occurring protein or to a fragment thereof, or it may contain mutations compared to the naturally-occurring sequence, provided that it retains ligand-binding ability.

Other human adenosine receptors include adenosine A_{2B}, A_3 and A_1 receptors. Thus the mutant GPCR may be a mutant adenosine A_{2B} receptor or a mutant adenosine A_3 receptor or a mutant adenosine A_1 receptor in which one or more amino acids corresponding to any of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 has been replaced by another amino acid.

The inventors have shown that mutating any of residues Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 increases the stability of a particular conformation (eg agonist). In other words the stability of that conformation is increased relative to the stability of that same conformation in the parent GPCR. Thus, the method of the invention may be considered to be a method for producing mutants of a GPCR which have increased stability of a particular conformation, for example they may have increased conformational stability. The methods of the invention may therefore be used to create stable, conformationally locked GPCRs by mutagenesis. The mutant GPCRs are effectively purer forms of the parent molecules in that a much higher proportion of them occupies a particular

conformational state. In an embodiment, the one or more mutants of the parent GPCR have increased stability in an agonist or antagonist conformation. It is appreciated that the methods of the invention may also be considered to be a method for producing mutant GPCRs which are more tractable to crystallisation. All such mutant GPCRs are included in the scope of the invention.

Conveniently, the method of the second aspect of the invention is performed and the stability of the resulting one or more mutants assessed. Methods for assessing the stability of GPCRs are known in the art and are described, for example, in WO 2008/114020 and in WO 2009/071914. Preferably, it is determined whether the resulting one or more mutants when residing in a particular conformation have increased stability with respect to binding a ligand (the ligand being one which binds to the parent GPCR when the parent GPCR is residing in a particular conformation), compared to the stability of the parent GPCR when residing in the same particular conformation with respect to binding that ligand. It is appreciated that the comparison of stability of the one or more mutants is made by reference to the parent molecule under the same conditions.

Since there are potentially thousands of mutations that can be screened in a GPCR for increased stability, it is advantageous to target particular mutations which are known to be important in conferring stability. Therefore, it will be appreciated that the method of the second aspect of the invention may also be used as a method of selecting mutant GPCRs with increased stability. The resulting one or more mutants can then be tested for increased stability, and those that have increased stability selected.

It is appreciated that the method of the second aspect of the invention may be repeated, for example once the stability of the resulting one or mutants are assessed, with the resulting one or more mutants generated in the first round becoming the parent GPCR in a subsequent round. Thus, the method can be used in an iterative way by, for example, carrying out a method to select mutant GPCRs with single mutations which have increased stability, combining those mutations in a single mutant GPCR, which then becomes the parent GPCR that is mutated in a subsequent round.

The mutant GPCR may be one which has increased stability to any denaturant or denaturing condition such as to any one or more of heat, a detergent, a chaotropic agent or an extreme of pH.

In relation to an increased stability to heat (ie thermostability), this can readily be determined by measuring ligand binding or by using spectroscopic methods such as fluorescence, CD or light scattering at a particular temperature. Typically, when the GPCR binds to a ligand, the ability of the GPCR to bind that ligand at a particular temperature may be used to determine thermostability of the mutant. It may be convenient to determine a "quasi T_m" ie the temperature at which 50% of the receptor is inactivated under stated conditions after incubation for a given period of time (eg 30 minutes). Mutant GPCRs of higher thermostability have an increased quasi Tm compared to their parents. Alternatively, thermostability can be assessed by measuring stability at a given temperature as a function of time. For example, the length of time at a given temperature by which the level of ligand binding falls to 50% of the level of ligand binding at time zero may be determined (Shibata *et al*, 2009 *J Mol Biol*). In either case however, it is appreciated that temperature is the denaturant.

In relation to an increased stability to a detergent or to a chaotrope, typically the GPCR is incubated for a defined time in the presence of a test detergent or a test chaotropic agent and the stability is determined using, for example, ligand binding or a spectroscopic method as discussed above.

In relation to an extreme of pH, a typical test pH would be chosen (eg in the range 4.5 to 5.5 (low pH) or in the range 8.5 to 9.5 (high pH).

Because relatively harsh detergents are used during crystallisation procedures, it is preferred that the mutant GPCR is stable in the presence of such detergents. The order of "harshness" of certain detergents is DDM, $C_{11} \rightarrow C_{10} \rightarrow C_9 \rightarrow C_8$ maltoside or glucoside, lauryldimethylamine oxide (LDAO) and SDS. It is particularly preferred if the mutant GPCR is more stable to any of C_9 maltoside or glucoside, C_8 maltoside or glucoside, LDAO and SDS, and so it is preferred that these detergents are used for stability testing.

It is preferred that the mutant GPCR has increased thermostability compared to its parent protein. It will be appreciated that heat is acting as the denaturant, and this can readily be removed by cooling the sample, for example by placing on ice. It is believed that thermostability may also be a guide to the stability to other denaturants or denaturing conditions. Thus, increased thermostability is likely to translate into stability

in denaturing detergents, especially those that are more denaturing than DDM, eg those detergents with a smaller head group and a shorter alkyl chain and/or with a charged head group. We have found that a thermostable GPCR is also more stable towards harsh detergents.

When an extreme of pH is used as the denaturing condition, it will be appreciated that this can be removed quickly by adding a neutralising agent. Similarly, when a chaotrope is used as a denaturant, the denaturing effect can be removed by diluting the sample below the concentration in which the chaotrope exerts its chaotropic effect.

In a further embodiment of the method of the second aspect of the invention, it is determined whether the mutant GPCR is able to couple to a G protein or another protein known to interact with a GPCR, for example a signalling protein such as arrestin or a GPCR kinase. Preferably, it is also determined whether the mutant GPCR is able to bind a plurality of ligands of the same class (eg agonist or antagonist), with a comparable spread and/or rank order of affinity as the parent GPCR.

The inventors have demonstrated that making mutations at the positions mentioned above in the amino acid sequence that defines a parent GPCR provides mutants of the parent GPCR that have increased stability. Thus, it is appreciated that the mutant GPCRs of the invention will have an extended lifetime, relative to its parent, under destabilising conditions.

Accordingly, it is appreciated that a third aspect of the invention provides a mutant GPCR which, which when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1), which mutant GPCR has increased stability compared to a parent GPCR when exposed to a destabilising condition of the parent GPCR with increased stability.

Similarly, the invention also provides a mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid (i) at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65,

Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1), which mutant GPCR has increased stability compared to a parent GPCR when exposed to a destabilising condition. Thus, the mutant GPCR may have increased stability in a particular conformation compared to a parent GPCR residing in the same conformation, when exposed to a destabilising condition.

It also appreciated that the invention allows for the production of compositions comprising mutant GPCRs, characterised in that the mutant GPCR is exposed to a destabilising condition. Such compositions have various applications, for example in crystallisation, drug screening, bioassay and biosensor applications.

Thus, a fourth aspect of the invention provides a composition comprising a mutant GPCR, which when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1), characterised in that the mutant GPCR is exposed to a destabilising condition effective to destabilise a parent GPCR to a greater extent than the mutant GPCR.

Similarly, the invention also provides a composition comprising a mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid (i) at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315,

according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1), characterised in that the mutant GPCR is exposed to a destabilising condition effective to destabilise a parent GPCR to a greater extent than the mutant GPCR.

By "destabilising condition" we include any condition which is capable of shifting the equilibrium of a population of GPCR proteins from the folded native state in a membrane to the unfolded state. In this way, the proportion of GPCR proteins existing in the unfolded state is increased and the proportion existing in the folded native state in a membrane is decreased.

By "population" we include a plurality of the same specific type of GPCR, as opposed to a mixture of different GPCRs. For example, the population may comprise at least 2, 5, 10, 50, 100, 200, 500, 1000, 5000, 10000, 100000, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} or 10^{14} GPCR molecules. Preferably, the population may comprise GPCR 10^9 and 10^{12} GPCR molecules.

In the folded native state in a membrane, GPCRs exhibit a biological activity, for example a binding activity or a signalling pathway modulation activity. Upon increasing exposure to a destabilising condition as described above, the equilibrium shifts further towards the unfolded state and an increasingly higher proportion of the GPCRs exist in the unfolded state. This change in structure from a folded to an unfolded state leads to a detectable change in the structure of the GPCR population. Moreover, this change in structure may lead to a detectable decrease in a biological activity of the GPCR population.

Accordingly in one embodiment, the destabilising condition is one that is effective to bring about a significant perturbation in the structure of a GPCR population compared to the structure of that population in the absence of the destabilising condition.

By a "significant perturbation in the structure of a GPCR population", we mean a perturbation which, when assessed relative to the statistical variation of the measurements used to detect the perturbation, would arise by chance in less than 1 in 10 measurements, more preferably 1 in 20 measurements and even more preferably 1 in 50 or 1 in 100 measurements.

Various methods to probe protein structure are known in the art and any suitable method may be used. For example, structural perturbations may be assayed by probing conformation directly e.g. with covalently attached fluorescent labels or esr spin labels, or by measuring the accessibility of native or deliberately introduced amino acid side chains within the protein population (Hubbell, W.L. et al., Adv. Protein. Chem. 63, 243-290 (2003); Baneres, J.L. et. al., J. Biol. Chem. 280, 20253-20260 (2005); Kobilka, B.K. and Deupi, X. Trends. Pharmacol. Sci. 28, 397-406 (2007)). For example, changes in fluorescence spectra, can be a sensitive indicator of protein unfolding, either by use of intrinsic tryptophan fluorescence or the use of the thiol specific fluorochrome N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (cpm) that becomes fluorescent upon reacting with cysteine residues exposed upon protein unfolding (Alexandrov *et al* (2008) Structure 16: 351-359). Proteolytic stability, deuterium/hydrogen exchange measured by mass spectrometry or nuclear magnetic resonance spectroscopy, blue native gels, capillary zone electrophoresis, circular dichroism (CD) or linear dichroism (LD) spectra and light scattering may also be used to measure structural perturbation by loss of signals associated with secondary or tertiary structure.

In another embodiment, the destabilising condition is one that is effective to bring about a significant reduction in a biological activity of a GPCR population (e.g. binding activity or signalling pathway modulation activity), compared to the level of the same activity in the absence of the destabilising condition. For example, the agent may be one that reduces the biological activity of a GPCR population to 90 – 10%, such as 70 – 30% or 60 – 40% of the level of the same activity when measured in the absence of the destabilising condition.

Depending upon the biological activity, it will be appreciated that the activity of the GPCR population may be measured using any suitable method known in the art.

By 'binding activity', we include binding to any binding partner that is known to bind to the GPCR. For example, the binding partner may be a ligand, for example one which causes the GPCR to reside in a particular conformation, or it may be an antibody, for example a conformational-specific antibody. Binding activity can be assessed using routine binding assays known in the art. Conveniently, the binding partner is detectably labelled, eg radiolabelled or fluorescently labelled. Alternatively, binding can be assessed by measuring the amount of unbound binding partner using a secondary detection system, for example an antibody or other high affinity binding partner

covalently linked to a detectable moiety, for example an enzyme which may be used in a colorimetric assay (such as alkaline phosphatase or horseradish peroxidase). Biophysical techniques such as patch clamping, fluorescence correlation spectroscopy, fluorescence resonance energy transfer and analytical ultracentrifugation may also be used (as described in New, R. C., *Liposomes: a practical approach*. 1st ed.; Oxford University Press: Oxford, 1990, and Graham, J. M.; Higgins, J. A., *Membrane Analysis*. Springer-Verlag: New York, 1997.)

Where the biological activity is a signalling pathway modulating activity, the activity can be assessed by any suitable assay for the particular signalling pathway. The pathway may be downstream of G protein activation or may be independent of G protein activation. Activation of the G protein can be measured directly by binding of a guanine nucleotide such as radiolabelled GTP to the G protein (Johnson et al, Assay Drug Dev Technol. 2008 Jun;6(3):327-37). Alternatively, binding of a signalling protein such as a G protein or an arrestin to the receptor may be measured by fluorescence resonance energy transfer (FRET) (Lohse et al, Adv Protein Chem. 2007;74:167-88) or related assays such as bioluminescence resonance energy transfer (BRET) (Gales et al, Nat Methods. 2005 Mar;2(3):177-84) or an enzyme complementation assay (Zhao et al, J Biomol Screen. 2008 Sep;13(8):737-47. Epub 2008). These assays are commonly available in kits for example from Perkin Elmer or CisBio or DiscoverX. The activity may be measured by using a reporter gene to measure the activity of the particular signalling pathway. By a reporter gene we include genes which encode a reporter protein whose activity may easily be assayed, for example β-galactosidase, chloramphenicol acetyl transferase (CAT) gene, luciferase or Green Fluorescent Protein (see, for example, Tan *et al,* 1996 *EMBO J* 15(17): 4629-42). Several techniques are available in the art to detect and measure expression of a reporter gene which would be suitable for use in the present invention. Many of these are available in kits both for determining expression *in vitro* and *in vivo*. Alternatively, signalling may be assayed by the analysis of downstream targets. For example, a particular protein whose expression is known to be under the control of a specific signalling pathway may be quantified, or a secondary metabolite may be quantified. Protein levels in biological samples can be determined using any suitable method known in the art. For example, protein concentration can be studied by a range of antibody based methods including immunoassays, such as ELISAs, western blotting and radioimmunoassay or by the use of biosensors (Ponsioen et al EMBO Rep. 2004 Dec;5(12):1176-80).

In an embodiment, the destabilising condition is any of heat, a detergent, a chaotropic agent such as guanidinium thiocyanate, an extreme of pH, an organic solvent, an aqueous solution or a membrane free environment.

For example, the destabilising condition may be a detergent, including for example, detergents that are of interest for subsequent crystallisation studies, for instance short chain-length detergents with a high CMC, such as C8-glucoside, C8-thioglucoside, C9-glucoside, C8-maltoside, C8-thiomaltoside, C9-maltoside, C9-thiomaltoside, Cymal 5, C8E5, or lauryl dimethylamine oxide. Short chain-length detergents are more likely to allow the formation of a 3-dimensional crystal lattice, and are easier to remove from receptor preparations by dialysis or other means than are long chain-length detergents with low CMCs.

It will also be appreciated that the destabilising condition may be any other amphiphilic molecule. For example, the destabilising condition may be any of amphipols, amphiphilic peptides such as mellitin, proteins such as apolipoproteins and their derivatives, organic solvents such as trifluoroethanol, dimethylformide, dimethylsulphoxide and chloroform/methanol mixtures, urea, a cylcodextrin, poly-ene antibiotics, guanidine hydrogenchloride, local anaesthetics and drugs such as procaine and chlorpromazine, polyols such as butane diol and heptane triol, short chain alcohols such as ethanol, propanol, isopropanol, butane diol and benzyl alcohol.

It will also be appreciated that the destabilising condition may be any aqueous solution.

It will further be appreciated that the destabilising condition may be a membrane free environment, such that the mutant GPCR exists in a form that is membrane free as discussed below.

In any event, the destabilising condition is one which is capable of shifting the equilibrium of a population of GPCR proteins from the folded native state in the membrane, to the unfolded state.

In one embodiment of the third and fourth aspects of the invention, the mutant GPCR is membrane free. By 'membrane free' we include the meaning of the mutant GPCR being substantially free of a membrane such as a lipid bilayer or a lipid monlayer. For

example, the mutant GPCR may be in a form where it does not reside within a membrane, unlike when it does reside in a membrane when in the native folded state.

In another embodiment, the mutant GPCR is in a non-aggregated form. By a "non-aggregated form" we include the meaning that the GPCR protein molecules are not aggregated, i.e. complexed with each other to form high molecular weight aggregates. By "aggregate" we include the meaning of a higher order molecular complex, e.g., a complex that comprises two or more tetramers of the protein. The molecular weight of such aggregates typically exceeds about 100 kDa, and more typically about 150 kDa. Aggregates are distinguished from multimers, where the term "multimer" refers to oligomers, such as dimers, trimers, and tetramers. Non-aggregating GPCRs of the invention include GPCRs that show reduced aggregation in vitro and/or in vivo as compared to their corresponding aggegrating parent GPCRs.

Given the increased stability of a mutant GPCR according to the invention, it is appreciated that the destabilising condition will destabilise the parent GPCR to a greater extent than the mutant GPCR, i.e. shift the equilibrium of a population of the parent GPCR from the folded native state to the unfolded state, further than it shifts the equilibrium of a population of the mutant GPCR from the folded native state in a membrane to the unfolded state.

Thus, when the parent GPCR manifests, for example, 50% of a biological activity when exposed to a destabilising condition, typically, the mutant GPCR with increased stability relative to the parent protein, will have at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% more biological activity than the parent protein when exposed to the destabilising condition, and more preferably at least 60%, 70%, 80%, 90% or 100% more activity, and yet more preferably at least 150% or 200% more activity.

Similarly, a mutant GPCR with increased stability relative to the parent GPCR will have a structure that is more similar to the folded-native state than the structure of the parent protein is to the folded-native state, when exposed to a destabilising condition.

In this way, the invention allows for mutant GPCRs that have increased stability compared to a parent GPCR when exposed to a destabilising condition, and for compositions comprising a mutant GPCR of the invention, characterised in that the mutant GPCR is exposed to a destabilising agent effective to destabilise a parent GPCR

to a greater extent than the mutant GPCR. For example, the invention allows for a solubilised form of a mutant GPCR of the invention.

Preferences for the mutant GPCRs in the third and fourth aspects of the invention include those defined above with respect to the first aspect of the invention. For example, the mutant GPCR of the third aspect of the invention may be, or the composition of the fourth aspect of the invention may comprise, a mutant adenosine receptor which, when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to one or more of Ser 47, Ala 50, Phe 62, Thr 65, Phe 79, Phe 83, Gln 89, Ser 90, Ala 236, Ile 238, Phe 242 and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1). Similarly, the mutant GPCR of the third aspect of the invention may be, or the composition of the fourth aspect of the invention may comprise a mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid (i) at a position which corresponds to any one or more of Serine 47, Alanine 50, Phenylalanine 62, Threonine 65, Phenylalanine 79, Phenylalanine 83, Glutamine 89, Serine 90, Alanine 236, Isoleucine 238, Phenylalanine 242 and Valine 282, and (ii) at a position which corresponds to any one or more of Leucine 48, Alanine 54, and Valine 57, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1). Preferably, the adenosine receptor has an amino acid sequence which is at least 20% identical to that of the human adenosine receptor whose sequence is set out in Figure 7 (SEQ ID No: 1).

It is preferred that the mutant GPCRs of the invention, or the mutant GPCRs within the composition of the invention, have increased stability to any one of heat, a detergent, a chaotropic agent and an extreme of pH. Preferably, the mutant GPCR has increased stability (e.g. thermostability) compared to its parent when in the presence or absence of a ligand thereto. Typically, the ligand is an antagonist, a full agonist, a partial agonist or an inverse agonist, whether orthosteric or allosteric. The ligand may be a polypeptide, such as an antibody.

It is preferred that the mutant GPCR of the invention, or the mutant GPCR within the composition of the invention, is at least 1°C or 2°C more stable than its parent, preferably at least 5°C more stable, more preferably at least 8°C more stable and even more preferably at least 10°C or 15°C or 20°C more stable than its parent. Typically, thermostability of the parent and mutant receptors are measured under the same

conditions. Typically, thermostability is assayed under a condition in which the GPCR resides in a particular conformation. Typically, this selected condition is the presence of a ligand which binds the GPCR.

It is preferred that the mutant GPCR of the invention, or the mutant GPCR within the composition of the invention, when solubilised and purified in a suitable detergent has a similar thermostability to bovine rhodopsin purified in dodecyl maltoside; however it is appreciated that any increase in stability will be useful for applications such as crystallisation studies. It is preferred that the mutant GPCR of the invention, or the mutant GPCR within the composition of the invention retains at least 50% of its ligand binding activity after heating at 40°C for 30 minutes. It is further preferred that the mutant GPCR of the invention, or the mutant GPCR within the composition of the invention retains at least 50% of its ligand binding activity after heating at 55°C for 30 minutes.

The mutant GPCRs and compositions disclosed herein are useful for crystallisation studies and are useful in drug discovery programmes. Thus it is appreciated that the invention provides a mutant GPCR in a crystallised form, optionally wherein the mutant GPCR is bound to a ligand such as an agonist or antagonist. The mutant GPCRs may be used in biophysical measurements of receptor/ligand kinetic and thermodynamic parameters eg by surface plasmon resonance or fluorescence based techniques. They may be used in ligand binding screens, and may be coupled to solid surfaces for use in high throughput screens or as biosensor chips. Biosensor chips containing the mutant GPCRs or compositions may be used to detect molecules, especially biomolecules.

The invention also includes a polynucleotide which encodes a mutant GPCR of the invention. In particular, polynucleotides are included which encode the mutant adenosine, mutant serotonin or mutant muscarinic receptors of the invention. The polynucleotide may be DNA or it may be RNA. Typically, it is comprised in a vector, such as a vector which can be used to express the said mutant GPCR. Suitable vectors are ones which propagate in and/or allow the expression in bacterial or mammalian or insect cells.

The invention also includes host cells, such as bacterial or eukaryotic cells, which contain a polynucleotide which encodes the mutant GPCR. Suitable cells include *E. coli* cells, yeast cells, mammalian cells and insect cells.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

The invention will now be described in more detail with respect to the following Figures and Examples wherein:

Figure 1: Positions of thermostabilising mutations in the primary sequence of the human A_{2A} receptor. The snake plot depicts the secondary structure elements found in the structure of the A_{2A} receptor, with the approximate position of the lipid bilayer shown in grey. (a) Thermostabilising mutations in the ^{3}H-NECA-bound conformation are shown in orange. Mutations identified previously from ^{3}H-NECA assays performed after heating the unliganded receptor are shown in red[23]. Mutations that were selected by both assays are blue. (b) The 16 most thermostabilising mutations of the ^{3}H-NECA-bound conformation of $A_{2A}R$ were re-assayed for thermostability in the antagonist-bound conformation using ^{3}H-ZM241385 (Table 1): yellow, mutants that did not bind antagonist in this assay; green, mutants that are less stable than wild-type $A_{2A}R$ in the antagonist-bound conformation; brown, mutants that are more stable than wild-type in the antagonist-bound conformation.

Figure 2: Thermostability of agonist-bound DDM-solubilised $A_{2A}R$ and thermostabilised mutants. (a) Additive effect of thermostabilising mutations used to generate $A_{2A}R$-GL23. Predicted T_m values were calculated by adding the ΔT_m for each mutation (Table 1) to the apparent T_m of wild-type $A_{2A}R$. The dashed line correlates with perfect additivity. Wild-type $A_{2A}R$ (WT) is represented by the black diamond. The stabilities of the best thermostable mutants containing one, two or three point mutations are labelled, respectively: L48A (GL0), green diamond; L48A-Q89A (GL10), dark blue diamond; L48A-Q89A-T65A (GL23), red diamond. Other double mutants (light blue diamonds) and triple mutants (light red diamonds) that were less stable than the optimal combinations are also shown. (b) Thermostability assays were performed on receptors partially purified in 0.025% DDM and with ^{3}H-NECA bound; $A_{2A}R$ (black circles), apparent T_m 28.6 ± 0.2 °C, n=8; GL0 (green squares), apparent T_m 42.2 ± 1.0 °C, n=5; GL10 (blue triangles), apparent T_m 46.7 ± 0.4, n=3; GL23 (red inverted triangles), apparent T_m 49.9 ± 0.1, n=2. (c) Stability of mutants compared to wild-type $A_{2A}R$ based on $t_{1/2}$ values calculated from (b) to allow the improvement in stability of the mutants compared to wild-type to be

calculated: GL0, 49-fold; GL10, 136-fold; GL23, 226-fold. The colour code is the same as in (b).

Figure 3: Thermostability of the mutants GL23 and GL26. (a) Thermostability of GL26 in (red squares; apparent T_m=44.5 ± 0.8°C, n=3) and GL23 (blue circles, apparent T_m=42.2 ± 0.3°C, n=2) partially purified in DM (0.17%), both with ^{3}H-NECA bound. (b) The thermostability of GL26 with ^{3}H-NECA bound was determined by partially purifying the receptor in different detergents. GL26 was solubilised in DM, immobilised on Ni^{2+}-NTA agarose and then detergent exchange was performed. The results are from a single experiment performed in triplicate, with the final concentration of detergent indicated: red squares, 0.17% decylmaltoside, apparent T_m 42.0°C; pink squares, 0.39% hega-10, apparent T_m 42.3°C; green inverted triangles, 0.3% nonylglucoside, apparent T_m 34.6°C; blue triangles, 0.52% foscholine-10, apparent T_m 33.1°C; orange circles, 0.42% octylthioglucoside, apparent T_m 30.5°C; pale blue circles, 0.37% polyoxyethylene C8E4, apparent T_m 26.4°C.

Figure 4: Affinities of agonists and antagonists for $A_{2A}R$ and the thermostabilised mutants. (a,b) Competition binding experiments were performed by measuring the displacement of ^{3}H-NECA bound to receptors in CHO cell membranes. Experiments were performed using three agonists (NECA, ATL146e, CGS21680) and three antagonists (ZM241385, CGS15943, SCH58621) with example curves shown for ZM241385 (a) and NECA (b); wild-type $A_{2A}R$, black circles; GL0 (green squares), GL23 (blue triangles) and GL26 (red inverted triangles). Full data are shown in Table 3. (c) The differences in affinities (ΔpK_i) between the wild-type $A_{2A}R$ and each of the mutants were calculated from the pK_i values determined in Table 3; GL0 (green), GL23 (blue) and GL26 (red).

Figure 5: Purification of $A_{2A}R$-GL31. The unglycosylated mutant of $A_{2A}R$-GL26, GL31, was expressed in insect cells using a recombinant baculovirus and purified on Ni^{2+}-NTA. The receptor was further purified by size exclusion chromatography (A_{280} trace shown; void (V_0) and total (V_T) column volumes indicated); this gave a symmetrical peak, which indicated that the preparation was monodisperse and homogenous. A coomassie-blue-stained SDS-polyacrylamide gel (inset) showed that $A_{2A}R$-GL31 represented a single band on the gel (left-hand lane) that was sufficiently pure for crystallisation (molecular weight markers are shown to the right).

Figure 6: Positions of the thermostabilising mutations in the antagonist-bound A_{2A} structure. The structure of A_{2A}R-StaR2 (PDB code 3PWH) thermostabilised in an antagonist-bound conformation is shown in rainbow colouration with the N-terminus and C-terminus labelled (N and C, respectively) and the bound antagonist ZM241385 depicted as a space-filling model (C, pink: N, blue; O, red). The four amino acid residues mutated in A_{2A}R to generate the thermostable mutant GL26 (L48A, Q89A, T65A and A54L) are depicted as space-filling models (grey). Note that the amino acid sequence shown is that of A_{2A}R-StaR2, which also contains the A54L thermostabilising mutation, whereas Leu48, Gln89 and Thr65 are identical to wild-type A_{2A}R.

Figure 7: Amino acid sequence of human adenosine A_{2A} receptor (SEQ ID No: 1).

Example 1: Thermostabilisation of an agonist-bound conformation of the human adenosine A_{2A} receptor

Summary

The adenosine A_{2A} receptor (A_{2A}R) is a G protein-coupled receptor (GPCR) that plays a key role in transmembrane signalling mediated by the agonist adenosine. The structure of A_{2A}R was determined recently in the antagonist-bound conformation, which was facilitated by the T4 lysozyme fusion in cytoplasmic loop 3 and the considerable stabilisation conferred on the receptor by the bound antagonist ZM241385. Unfortunately, the natural agonist, adenosine, destabilises the receptor and diffraction-quality crystals are no longer obtained. As a first step towards determining the structure of A_{2A}R bound to an agonist, the receptor was thermostabilised by systematic mutagenesis in the presence of the bound agonist ^{3}H-NECA. Four thermostabilising mutations were identified that, when combined to give the A_{2A}R-GL26 mutant, conferred greater than 200-fold increased stability compared to the wild-type receptor. Pharmacological analysis suggested that A_{2A}R-GL26 is stabilised in an agonist-bound conformation because antagonists (ZM241385, CGS15943) bind with up to 320-fold decreased affinity. None of the thermostabilising mutations are in the ZM241385 binding pocket, suggesting that the mutations affect ligand binding by altering the conformation of the receptor rather than through direct interactions with ligands. A_{2A}R-GL26 shows considerable stability in short chain detergents, which has allowed its purification and crystallisation.

Methods

Expression of adenosine A_{2A} receptor point-mutants in Escherichia coli

The library of Ala/Leu scan mutants in the receptor A_{2a}R-(2-316) was expressed from plasmid pRG/III-hs-MBP in *E. coli* strain DH5α as previously described[23]. Cells were grown at 37 °C in 2L flasks containing 500 mL of 2 × TY medium supplemented with Ampicillin (100 μg/mL) and glucose (0.2% w/v). At OD_{600} of 0.7, IPTG and theophylline were added at final concentrations of 0.5 mM and 100 μM, respectively, and the temperature was reduced to 20 °C. After 22 to 24 hours, cells were harvested in aliquots of 14 mL, centrifuged (30 minutes, 5,000 g) and stored at -20°C.

Solubilisation and partial purification of adenosine A_{2A} receptor mutants for thermostability assays

An aliquot of *E. coli* cells (14 mL) was thawed on ice and resuspended in 500 μL of buffer A [50 mM Tris HCl pH 7.4; 0.4 M NaCl, 250 μg/mL lysozyme (Sigma), 1 mg/mL DNAse I (Sigma), supplemented with complete EDTA-free Protease inhibitor cocktail (Roche)] and incubated 1 hour at 4 °C. Samples were then sonicated for 1 minute at 4°C using a cup-horn sonicator. The receptors were solubilised by adding 1% DDM and incubated for 1 hour at 4°C. Insoluble material was removed by centrifugation (5 minutes, 13000 *g*, 4°C). The solubilised receptors were partially purified with Ni-NTA agarose (Qiagen). Agarose beads (300 μL) pre-equilibrated in buffer A were added to the 700 μL of solubilised receptor. To reduce the detergent concentration, a solution of 50 mM Tris HCl pH 7.4; 0.4 M NaCl was added to a final volume of 2 mL. After 2 hours of incubation at 4°C, samples were centrifuged (13000*g*, 10 seconds, 4°C) and washed 3 times in buffer B (25mM Hepes pH 7.4, 0.025% DDM) and then eluted in buffer B supplemented with 50 mM histidine, for 30 minutes at 4°C. The supernatant was used directly in radioligand-binding assay.

Radioligand binding assay and thermostability assay for detergent solubilised receptors

108 μL of solubilised receptor was mixed with 12 μL of 4 μM ^{3}H-NECA (final concentration of 400 nM) or 12 μL of 1 μM ^{3}H-ZM241385 (final concentration of 100 nM). The sample was incubated for 45 min at 4°C, then 30 min at the specified temperature

and then 30 min at 4°C. Receptor bound and free radioligand were separated as previously described on mini gel filtration columns[19]. Receptor-bound ligand were transferred to a 96-well plate (Perkin Elmer) and mixed with 200 μL of Optiphase supermix (Perkin Elmer). The bound ^{3}H-ligand was determined using a 1450 Microbeta Trilux counter (1 min per sample).

Mammalian Cell culture and receptor expression

Chinese hamster ovary (CHO) cells were maintained in culture in DMEM HAMs F12 media containing 10 % FBS. Cells were transfected with either wild-type adenosine A_{2A} receptor or a stabilised receptor construct using GeneJuice according to the manufacturer's instructions. After 48h post-transfection, cells were harvested by scraping and centrifuged (200 g, 5 minutes, 4° C). The supernatant was removed and the pellet re-suspended in 10 mL 20 mM HEPES buffer + 10 mM EDTA buffer (pH 7.4). The membrane suspension was homogenised (10 s; 20500 rpm) and centrifuged (200 g, 15 min, 4° C). The supernatant was collected, the pellet re-suspended in 10 mL HEPES/EDTA buffer and the solution homogenised and centrifuged as described before. The collected supernatant was centrifuged (30 minutes, 40000 g, 4°C). Pellets were re-suspended in 20 mM hepes pH 7.4, 0.1 mM EDTA to a concentration of 1 mg/ml and stored at -80°C until further use.

Ligand binding assays, saturation and competition binding experiment

Membranes from CHO cells transiently expressing receptors (10-15 μg / well) were assessed using competition ^{3}H-NECA binding in buffer containing 50 mM Tris-HCl (pH 7.4). Non-specific binding was defined using 1 μM CGS21680. After 1 h incubation at 25°C, assays were terminated by filtration through 96-well GF/B filter plates pre-soaked with 0.1 % PEI and washed with 5 x 0.5 mL water. Plates were dried and bound ligand measured using a Microbeta counter. Inhibition curves were fitted to a four-parameter logistic equation to determine IC_{50} values which were converted to K_i values using K_D values determined by saturation binding and the ^{3}H-NECA concentration (~10 nM).

Purification of NECA-bound A_{2A}R-GL31

Receptors were expressed with the baculovirus system using Tni cells (High 5™) and the vector pBacPAK8 (Invitrogen). Insect cells were grown in suspension in a maximum

volume of 500 ml in 2 L roller bottles (Corning) at 27°C with shaking at 150 rpm. Sf9 cells were grown in TNM-FH medium supplemented with 10% FBS and Tni cells were grown in EXcell 405 medium supplemented with 5% FBS (heat inactivated); all media were supplemented with 1% lipids (Invitrogen). The GL31 construct was inserted into plasmid pBacPAK8 using the restriction enzyme *BamHI/XbaI*. Sf9 cells were used to generate the first virus passages, and to obtained 2nd and 3rd passage high-titre virus stocks. Tni cells were grown to 2-2.5 x 10^6 cells/L, diluted in a 1:1 volume ratio with fresh media and infected with the recombinant baculovirus. Cells were harvested 72 hours post-infection, resuspended in 25 mM Hepes pH 7.4, 1x protease inhibitor cocktail and snap-frozen in liquid N_2.

All protein purification steps were performed at 4°C. Frozen cell pellets equivalent to 2 L of cell culture were thawed and resuspended at room temperature in 25 mM Hepes pH 7.4, EDTA 1 mM, PMSF (0.5 mM), Pepstatin (1μg/ml) and Leupeptin (1μg/ml) or 1x protease inhibitor cocktail, to give a final volume of 360 ml. The cells was centrifuged (120,000 g, 2 hours, 4°C) to pellet the washed cells and membranes, the supernatant was carefully removed and the pellet resuspended in 240 mL of the same buffer. Cells were homogenised using a Polytron (12000 rpm, 2 x 15 seconds on ice) and centrifuged to pellet the membranes (45,000 g, 2 hours, 4°C). The pellet was resuspended in 25 mM Hepes pH 7.4, PMSF (0.5 mM), Pepstatin (1μg/ml) and Leupeptin (1μg/ml) or 1x protease inhibitor cocktail, homogenised using a Polytron (12000 rpm, 2 x 15 seconds on ice) and snap-frozen in liquid N_2.

Membranes were thawed at room temperature, diluted with 25 mM Hepes pH 7.4, PMSF (0.5 mM), Pepstatin (1μg/ml) and Leupeptin (1μg/ml) or 1x protease inhibitor cocktail 100 ml. Membranes were pre-incubated with NECA at 100 μM for 45 minutes before solubilisation. Receptors were solubilised by adding DM and NaCl to give final concentrations of 1.5% and 0.3 M, respectively, followed by centrifugation (120,000 g, 45 minutes, 4°C). The solubilised receptor sample was then filtered through a 0.22 μm filter (Millipore) and applied at 0.3 ml/min to a 5 ml Ni-NTA superflow cartridge (Qiagen) pre-equilibrated with buffer (25 mM Hepes pH 7.4, 0.1M NaCl, 100 μM NECA, 0.15% DM, 2.5 mM imidazole). The column was washed (1 ml/min) with the same buffer supplemented with either 10, 40 or 80 mM imidazole for 5, 10, 5 column volumes, respectively, and then eluted with 5 column volumes of elution buffer (25 mM Hepes pH 7.4, 0.1 M NaCl, 100 μM NECA, 0.15% DM, 250 mM imidazole). The eluted receptor was

mixed with TEV protease to cleave the tag for 4-6 hours, 4°C. After cleavage, 14-16 ml of the pooled fractions were concentrated to 2 ml using an Amicon-ultra spin concentrator (Ultracel-50K, Millipore) and loaded onto a PD-10 column (GE Healthcare) in order to remove the imidazole. A negative purification was used to remove the TEV protease by loading the sample in batch onto 5 ml Ni-NTA (QIAGEN) pre-equilibrated in 25 mM Hepes pH 7.4, 0.1 M NaCl, 100 μM NECA, 0.15% DM, 40 mM imidazole, and incubated for 30 minutes. The resin was spun down and the supernatant containing the receptor removed. For detergent exchange (into, for example, 0.35% NG), the sample (5.5-6 ml) was concentrated down to 0.5 ml using an Amicon-ultra concentrator (Ultracel-50K, Millipore), diluted 10-fold in 25 mM Hepes pH 7.4, 0.1 M NaCl, 100 μM NECA, 0.35% NG, and concentrated down again to 0.3 to 0.5 ml. The protein sample was applied to a 10/30 S200 size exclusion column pre-equilibrated in 25 mM Hepes pH 7.4, 0.1 M NaCl, 100 μM NECA, 0.35% NG and run at 0.5 ml/minute. Protein determination was performed using the amido black assay[29].

Determination of receptor half life ($t_{1/2}$) using the Arrhenius law

For all the thermostabilisation studies carried out in our lab, the apparent T_m is the temperature for which 50% of the solubilised receptor remains folded after 30 min incubation. The rate constant of the protein denaturation is a function of the temperature and consequently thermal denaturation of a protein can be compared to a chemical reaction for which the rate of the reaction is dependant of the temperature. Arrhenius established that any chemical reaction is temperature dependant. We used a simplified version of the Arrhenius law to estimate the $t_{1/2}$ of the A_{2A} receptor: $A= A_0 \exp(-0.693*t/t_{1/2})$ in which A is the total sample activity, A_0 the activity for a defined temperature, t the experimental time used for heating the sample and $t_{1/2}$ the half life of the A_{2A} receptor bound to its agonist NECA. From the experimentally determined T_m curves, the values t and A were selected as being the slope of the curve, defining a window around the T_m value, which represents the linear part of the curve, from 26.5°C to 32°C for the WT, 39°C to 45°C for GL0, 44°C to 50°C to GL10 and 47.5°C to 53°C for GL23. The reference measurement representing the total binding activity of the sample A_0 was considered to be measured at 4°C From the graphical representation $t= \text{Log}_{10}(t_{1/2})$ (Fig. 2c), we could extract a half-life for each construct at any temperature to define the improvement in stability of the various mutants compared to wild-type $A_{2A}R$.

Results

Extracellular adenosine influences cellular function throughout the body, particular during cellular stresses such as anoxia, but in the central nervous system it functions as a ubiquitous neuromodulator. There are four adenosine receptors found in humans, A_1, A_{2A}, A_{2B} and A_3, which belong to Family A of GPCRs and the A_1, A_{2A} and A_{2B} are well known for being inhibited by the antagonist caffeine. Adenosine receptors are implicated in the pathophysiology of many neurological disorders such as Parkinson's disease, Huntington's disease as well as ischemia (cerebral and cardiac), inflammatory and immune diseases. The adenosine A_{2A} receptor ($A_{2A}R$) is therefore a potential target for treating many diseases, with the most advanced drug, preladenant, currently in phase III clinical trials for the treatment of Parkinson's disease.

A structure of $A_{2A}R$ has been determined in the antagonist-bound conformation with the inverse agonist ZM241385 bound[7]. The structure determined was of a receptor T4 lysozyme (T4L) fusion protein crystallised in lipidic cubic phase (LCP). Intracellular loop 3 (IL3) of GPCRs is of variable length and is predicted to be flexible to allow coupling to the G protein. Engineering of T4L into this loop produced a chimaeric receptor, A_{2A}-T4L, which maintains its ability to bind both agonists and antagonists, although this modified receptor binds agonists with higher affinity compared to wild-type $A_{2A}R$[7]. A_{2A}-T4L was crystallised in the presence of the high-affinity inverse agonist ZM241385, which imparts considerable stability to detergent-solubilised $A_{2A}R$. When low-affinity agonists bind to GPCRs, the agonist-receptor complex is often less stable than the antagonist-receptor complex[14], which makes it more difficult to crystallise and produce well-diffracting crystals. Therefore, to obtain the structure of $A_{2A}R$ with an agonist bound, we have stabilised $A_{2A}R$ in a specific agonist-binding conformation.

Conformational thermostabilisation is a strategy used to engineer a membrane protein so that it is sufficiently stable in short chain detergent for crystallisation and structure determination (eg StaR technology). The strategy has been applied to the thermostabilisation of the β_1-adrenergic receptor (β_1AR)[15, 16], the neurotensin receptor (NTS1)[17] and to $A_{2A}R$[18]. In each instance it was observed that if the selection for thermostabilising mutations was performed with an antagonist, then the thermostabilised mutant was preferentially in an antagonist-binding conformation; similarly, selection with an agonist resulted in a receptor in the agonist-binding conformation. As there are distinctive structural differences between the agonist- and antagonist-binding

conformations, it is of little surprise that different mutations are required to stabilise the two different states[18]. Stabilisation of β_1AR and A_{2A}R in the antagonist-bound conformation allowed the crystallisation and structure determination of both receptors[8] (Dore *et al*, unpublished).

We now describe a strategy for the stabilisation of A_{2A}R in an agonist-bound conformation. The stabilised receptor A_{2A}R-GL26 was found to be extremely stable in short chain detergents when the agonist NECA was bound, which allowed its purification in a monodisperse state and its subsequent crystallisation.

Identification of thermostabilising point mutations in agonist-bound A_{2A}R.

The strategy used to isolate thermostabilising mutations involves making a library of mutants throughout the receptor where every amino acid residue is changed to alanine or, if the residue is already alanine, to leucine. Each mutant receptor is then expressed, solubilised in detergent and its thermostability determined.

The K_D for NECA binding to A_{2A}R is reduced by high concentrations of detergent and NaCl and the thermostability of ^{3}H-NECA-bound A_{2A}R is similarly reduced under these conditions[18]. Therefore, the thermostability assay developed contained low concentrations of DDM and no NaCl. After solubilisation in DDM, A_{2A}R was bound to Ni^{2+}-NTA resin, washed to reduce the DDM concentration and then eluted (see Methods). ^{3}H-NECA was then added to the sample, which was then heated at various temperatures for 30 minutes, quenched on ice and the receptor-bound ligand separated from free ligand on a mini-gel filtration column[19]. This assay was used to define the stability of the wild-type receptor (apparent T_m) and for the initial screen of all the Ala/Leu-scan mutants. The apparent T_m is defined as the temperature at which 50% of the solubilised receptor can still bind radioligand after a 30 minute incubation[15, 17, 18].

The apparent T_m for wild-type A_{2A}R in the ^{3}H-NECA thermostability assay (see Methods) is 28.6 ± 0.2 °C (n=8). The library of Ala/Leu mutants made throughout A_{2A}R[23] was then expressed, solubilised in DDM and a single-point thermostability assay performed on each mutant by heating the ^{3}H-NECA-bound receptor at 28°C for 30 minutes; the results of these assays were compared to the thermostability of the wild-type receptor. Out of the 315 mutants screened, 38 were found to increase the thermostability of ^{3}H-NECA-

bound $A_{2A}R$ by a minimum of 40% and, in addition, maintained a minimum expression level of 30%, both values compared to wild-type $A_{2A}R$ (Fig 1).

The stabilising mutations of the invention are different from the mutants described previously that stabilise the ligand-free $A_{2A}R$ selected with ^{3}H-NECA (Fig 1). The apparent T_m was determined for each of 38 mutants and the 16 most thermostabilising mutations (Table 1) were selected for further study.

The L48A mutant provided the greatest thermostabilisation of the ^{3}H-NECA bound $A_{2A}R$ conferring a 13.6°C improvement in stability (apparent T_m 42.2 ± 0.75 °C; Fig. 2). The remaining 11 mutants improved the thermostability of ^{3}H-NECA bound $A_{2A}R$ by between 2-6°C (Table 1). The 16 selected mutations are highly clustered in the primary amino sequence of $A_{2A}R$, with 7 mutations in transmembrane region 2 (TM2), 4 in TM3 and 3 in TM6, with only a single mutation in TM1 and TM7 (Fig. 1).

The thermostabilising mutants were then tested for their ability to thermostabilise $A_{2A}R$ when the antagonist ZM241385 was bound. The apparent T_m of ^{3}H-ZM241385-bound $A_{2A}R$ was 32.0 ± 0.1 °C (Table 1), which is 3.5°C more stable than ^{3}H-NECA-bound $A_{2A}R$ measured under identical conditions. The 15 mutants that showed the highest thermostabilities in the NECA-bound conformation were tested for thermostability when ZM241385 was bound. The results show that these 15 mutants can be categorised into three classes (Table 1). The first category contained mutants L48A, F62A, F79A and F242A, which did not bind ^{3}H-ZM241385 at 4°C in this experiment, suggesting a dramatic loss of affinity (see below for ligand binding experiments), and their thermostability with ^{3}H-ZM241385-bound was not determined. The second category contained mutants S47A, F83A and Q89A, which stabilised the NECA-bound conformation and destabilised the ZM241385-bound conformation. The third group is composed of the remaining mutations that stabilised both agonist- and antagonist-bound conformations (A50L, A54L, V57A, T65A, S90A, A236L, I238A and V282A).

Combining mutants to make the optimally thermostable agonist-bound receptor $A_{2A}R$-GL26

Given the significant increase in thermostability observed for $A_{2A}R$-L48A, this was re-named GL0 and it was used as the starting point for the construction of an optimally stable agonist-binding receptor for structural studies. 13 of the remaining

thermostabilising point mutations were individually combined with the L48A mutation (Table 2). Each of the double mutants was expressed and its thermostability measured and compared to the predicted T_m (ΔT_m for each single mutant (Table 2) summed with the apparent T_m for wild-type $A_{2A}R$). Five of the double mutants did not increase the thermostability compared to the L48A single mutant (Fig. 2). All of the remaining double mutants showed higher thermostabilities compared to GL0, with the best combination being L48A-Q89A ($A_{2A}R$-GL10) with an apparent T_m of 46.7 ± 0.4 (Fig. 2). This strategy was repeated using the double mutant GL10 as the starting point and adding the 5 mutations found to be additive in the previous round. GL10 containing the mutation F83A could not be made. One mutant showed an additive effect for the third mutation, L48A-Q89A-T65A ($A_{2A}R$-GL23), which had an apparent T_m of 49.9 ± 0.1°C (Fig. 2). It is interesting to note that all the mutations used to construct GL23 are in TM2 and TM3 and that none of the mutations in TM6 were additive with L48A (Fig. 1; Table 2).

To improve the thermostability of GL23 further, the detergent used in the thermostability assay was changed to a shorter-chain detergent, n-decyl-β-D-maltopyranoside (DM). As expected, reducing the size of the detergent decreased the stability of the receptor, with $A_{2A}R$-GL23 being 7 °C less stable in DM than in DDM (Fig. 3). After a last round of mutagenesis combining the additive mutants to GL23, it was found that the addition of A54L to make mutant GL26 gave the highest thermostability. $A_{2A}R$-GL26 displayed very good thermostability in DM (apparent T_m 44.5 ±0.8°C) and also excellent stability in a variety of other short chain detergents ideal for crystallography (Fig 3).

An alternative method for displaying the thermostability of the various mutants is shown in Fig 2c, which relates the logarithm of the rate of inactivation of the receptor to the temperature (see Methods). Provided that all the assays are performed under identical conditions, this can be used to estimate the factor by which a receptor is stabilised compared to the wild-type receptor. Data extracted from Fig 2b was therefore re-plotted in Fig. 2c and used to estimate that NECA-bound $A_{2A}R$-GL23 was about 230-times more stable than the NECA-bound wild-type $A_{2A}R$.

Pharmacological characterisation of thermostabilised mutants

To characterise how the thermostabilising mutations have affected the conformation of $A_{2A}R$, three of the mutants (GL0, GL23, GL26) were transiently expressed in CHO cells, the membranes purified and competition binding analyses performed using both agonists

and antagonists, in addition to saturation binding experiments performed with ^{3}H-NECA (Table 3). The changes in pK_i for each mutant and all ligands tested are summarised in Fig. 4. All the mutants bound the antagonists more weakly, with the most significant reductions in affinity observed for ZM241385, CGS15943 and SCH58621 (Fig. 4). In contrast, there was no significant change in affinity for the binding of all the agonists tested (NECA, ATL146e and CGS21680) (Fig. 4) except that NECA bound 3-times more tightly to GL23 than to wild-type $A_{2A}R$ (Table 3). The data show that the major influence on the conformation of the ultimate mutant $A_{2A}R$-GL26 is from the L48A mutation in GL0. This single point mutation accounted for 60-88 % of the reduction in antagonist affinity observed in GL26.

Purification of the thermostable mutant $A_{2A}R$-GL31

$A_{2A}R$-GL26 was expressed using the baculovirus expression system in insect cells, to give about 2-3 mg/L of cell culture, and then purified using a two step process, a Ni^{2+}-NTA column followed by a StrepII tag affinity column. However, purified GL26 consisted of two species differing in molecular weight by about 3 kDa, which is consistent with only a proportion of the receptor being N-glycosylated (data not shown). Therefore, the predicted N-glycosylation site Asn154 was mutated to Ala, to make $A_{2A}R$-GL31 (L48A-Q89A-T65A-A54L-N154A). GL31 was expressed in insect cells, purified on a Ni^{2+}-NTA column, followed by size exclusion chromatography (Fig 5). Even if the size exclusion column was run using the detergent nonylglucoside (NG), purified GL31 exhibited a symmetrical peak, which is indicative of a highly purified, monodisperse sample (Fig. 5). After concentration, one preparation yielded up to 1 mg of receptor from a 2 L culture and the receptor could be concentrated up to 20 mg/ml without significant aggregation. $A_{2A}R$-GL31 bound to NECA produced good quality crystals that diffract to 2.6 Å resolution (Lebon and Tate, unpublished data).

$A_{2A}R$ mutation	Ballesteros-Weinstein	Apparent T_m (°C) Agonist: ^{3}H-NECA	ΔT_m	Antagonist: ^{3}H-ZM241385	ΔT_m
WT	-	28.5	-	32	-
V12A	1.38	30.5	+2	n.d.(1)	n.d.
S47A	2.45	31	+2.5	25.5	-6.5
L48A	2.46	42.5	+14	0(2)	n.d.
A50L	2.48	31	+2.5	38.5	+6.5
A54L	2.52	33.5	+5	38	+6
V57A	2.55	34.5	+6	38	+6
F62A	2.60	31	+2.5	0	n.d.
T65A	2.63	33	+4.5	38	+6
F79A	3.27	31	+2.5	0	n.d.
F83A	3.31	30	+1.5	16.5	-15.5
Q89A	3.37	34.5	+6	26	-8
S90A	3.38	32	+3.5	32	0
A236L	6.38	31	+2.5	38	+6
I238A	6.40	31	+2.5	36	+4
F242A	6.44	31	+2.5	0	n.d.
V282A	7.47	30.5	+2	33	+1

Table 1. Thermostability of $A_{2A}R$ mutants with either agonist or antagonist bound. Values were determined from a single thermostability curve with values determined in triplicate with an estimated error of ± 0.5°C. These values were used to give a rank order of thermostabilisation for the different mutants. For final values for wild-type $A_{2A}R$ and L48A (GL0), please refer to Table 2. (1) Not determined. (2) No binding detected at antagonist concentration used.

			Apparent T_m in DDM (°C)		
	Mutant name	$A_{2A}R$ Mutations	Predicted	Single measurement*	Final values*
Wild-type $A_{2A}R$	-	-	-	-	28.6 ± 0.2 (n=8)
Single mutant	GL0	L48A	42.5	42.5	42.2 ± 1.0 (n=5)
Double mutant	GL1	L48A-V12A	45	45.5	
	GL4	L48A-A54L	47.5	46	
	GL5	L48A-V57A	48.5	41.5	
	GL6	L48A-F62A	45	45	
	GL7	L48A-T65A	47	45	
	GL8	L48A-F79A	45	45.5	
	GL9	L48A-F83A	44	43.5	
	GL10	L48A-Q89A	48.5	47.5	46.7 ± 0.4 (n=3)
	GL11	L48A-S90A	46	46	
	GL14	L48A-A236L	45	40.5	
	GL15	L48A-I238A	45	42.5	
	GL17	L48A-F242A	45	39.5	
	GL19	L48A-V282A	44.5	40	
Triple mutant	GL20	L48A-Q89A-V12A	49.5	46	
	GL21	L48A-Q89A-A54L	52	46	
	GL22	L48A-Q89A-F62A	49.5	45.5	
	GL24	L48A-Q89A-F79A	49.5	46	
	GL23	L48A-Q89A-T65A	51.5	50	49.9 ± 0.1 (n=2)
			Apparent T_m in DM (°C)		
Triple mutant	GL23	L48A-Q89A-T65A			42.2 ± 0.3 (n=2)
Quadruple mutant	GL25	L48A-Q89A-T65A-A50L	46	44.5	
	GL26	L48A-Q89A-T65A-A54L	49	46	44.5 ± 0.8 (n=3)
	GL27	L48A-Q89A-T65A-F83A	44.5	44	
	GL29	L48A-Q89A-T65A- S263A	44.5	44.5	

Table 2. Combinations of mutants tested for thermostabilising the NECA-bound conformation of $A_{2A}R$. *Values were determined initially from a single thermostability curve, with measurements performed in triplicate, to find the most thermostable mutants; replicate experiments were performed only for key mutants. Predicted T_m was calculated from the parental experimental T_m value (single or double mutants) and the value of the

ΔT_m for the single mutant tested. Experimental measurements were then compared with the predicted T_m and classified as (i) an additive effect when the experimental value is equal or similar to the predicted T_m (± 1.5 °C), (ii) non-additive when the experimental value is different from the predicted T_m. Quadruple mutants were tested in DM.

		pK_i (-Log M)			
		WT	GL0	GL23	GL26
Agonist	NECA	7.82±0.20	7.94±0.06	8.43±0.07*	8.13 ± 0.11
	ATL146e	7.95±0.17	7.96±0.01	8.25±0.01	7.85±0.08
	CGS21680	6.94±0.11	6.89±0.05	7.24±0.19	6.77±0.06
Antagonist	ZM241385	9.22±0.04	7.65±0.17***	6.89±0.06***	6.67±0.08***
	CGS15943	9.68±0.11	7.91±0.06***	7.49±0.10***	7.17±0.08***
	SCH58621	8.92±0.17	6.97±0.06***	7.10±0.12***	4.61±0.20***

Table 3. Comparison of affinities of agonist and antagonist binding to $A_{2A}R$ and thermostable mutants. Competition experiments were performed by displacement of ^{3}H-NECA from receptors transiently expressed in CHO cells. The pK_i values are the mean of three independent experiments performed in triplicate ± S.E.M. pK_i values were calculated from the IC_{50} using the Cheng-Prusoff equation and the following values for K_D (nM) for ^{3}H-NECA: WT, 13.45 ± 0.44; GL0, 6.39 ± 0.59***; GL23, 4.65 ± 0.67***; GL26, 6.33 ± 0.66***. P values were determined using a one-way ANOVA with Dunnett's post-hoc test: *, P<0.05; **, P<0.01; ***, P<0.001 with respect to the wild type $A_{2A}R$.

Discussion

Conformational thermostabilisation of GPCRs has proven to be a successful strategy for their structure determination when the receptor is stabilised in the antagonist state, with structures of both a β_1-adrenoceptor[8] and $A_{2A}R$ (Doré et al. unpublished) with antagonists bound being determined. The real value of the approach was recently highlighted by the structures of β_1AR bound to low-affinity agonists[13]. The other approaches that have also

given GPCR structures[20] rely on increasing the hydrophilic area of the receptor by binding an antibody fragment and/or with a T4 lysozyme fusion and on the thermostabilisation of the receptor from a high-affinity ligand with a slow off-rate[7; 11].

We have now identified further stabilising mutations that stabilise the agonist conformation of the adenosine A2a receptor. From this work, we have produced the mutant A_{2A}R-GL26 that is highly thermostable and has already been purified and crystallised. The mutant was selected by selection of ^{3}H-NECA-bound A_{2A}R in a thermostability assay. Stabilisation in both the unliganded and NECA-bound conformations were not simultaneously selected for. The mutant, A_{2A}R-GL26 is similar in stability upon detergent solubilisation to native rhodopsin or β_1AR-m23, both of which have been crystallised and their structures determined to high resolution.

The mutant, A_{2A}R-GL26 contains four mutations that thermostabilise A2aR in the agonist-bound conformation. The single mutation with the greatest thermostabilising effect was L48A, which increased the stability of NECA-bound A_{2A}R by 14.5°C. The strategy was to make a series of double mutants by adding the best thermostabilising mutants to L48A, testing their thermostability, picking the most thermostable double mutant, and then adding the next best single thermostabilising mutation. At each stage, only those single mutations that gave a near additive increase in thermostability were tested in the next round. Out of 13 double mutants constructed and tested for their thermostability, the L48A-Q89A mutant had the highest thermostability, which was only slightly lower than the apparent T_m predicted by adding the ΔT_m for Q89A to the apparent T_m for L89A. In a similar fashion, T65A was found to further thermostabilise the receptor, making the triple mutant GL23 (L48A-Q89A-T65A) with an apparent T_m of 50°C in DDM. We have sometimes found that the measurement of apparent T_m at high temperatures may be less accurate than desired due to non-specific protein aggregation in the sample, therefore we decided to carry out one more round of thermostabilisation in DM. As expected, the thermostability of GL23 was lower in DM than in DDM (Fig.3). Amongst the five mutations tested, the best additive effect was observed for the A54L mutant. The final mutant GL26 displays an apparent T_m of 44.5°C in 0.15% DM, which is similar to that observed for the thermostable mutant β_1AR-m23 (apparent T_m 48°C)[15]. A_{2A}R-GL26 also displayed considerable stability in relatively harsh detergents like FC10, HEGA-10 and OTG, which have been used to crystallise β_1AR-m23[21].

In previous thermostabilisation experiments, each receptor was stabilised in a particular conformation depending on whether an agonist or an antagonist was used for the selection of thermostable mutants. The conformation of A_{2A}R-GL26 was therefore assessed by performing ligand binding assays using both agonists and antagonists. Binding affinities for the antagonists ZM241385 and CGS15943 were reduced by 320-fold, whereas the only statistically significant change in agonist binding affinity was observed for NECA (3-fold), whereas the affinities for ATL146e and CGS21680 were similar to wild-type A_{2A}R. None of the mutations used to thermostabilise GL26 are in the ligand binding pocket, which suggests that they affect the global conformation of the receptor. The binding data are consistent with GL26 being in an agonist-binding conformation. However, it is very unlikely that the conformation is identical to the fully activated state, because it is expected that agonist affinity would be increased by a factor of 15-40 or more[22, 23]. It is thus anticipated that the receptor will represent a conformation along the activation pathway, between the R state and the R* state.

Analysis of the ZM241385-bound structure of $A_{2A}R^{7}$ showed that none of the thermostabilising mutants in A_{2A}R-GL26 make direct contact to the ligand (Fig 6). It is therefore likely that the introduction of the 4 thermostabilising mutations L48A, T65A, Q89A and A54L has induced a conformational change in the receptor and the binding data supports the view that the mutant is an agonist-binding conformation between R and R*. The single biggest effect on ligand binding was seen for the mutation $L48A^{2.46}$ (superscript refers to the Ballesteros-Weinstein numbering system[24]) with a 2-fold increase in the affinity for the agonist NECA and a 50-fold decrease in affinity for the antagonist ZM241385. In the entire GPCR family, $Leu^{2.46}$ is one of the most conserved residues in TM2 (approximately L 96%, M 2%, I 1.5% and V/T 0.5%). When mutated to alanine, $Leu^{2.46}$ displays constitutive activity in rhodopsin[25] and the thyrotropin receptor[31]. The side chain of $Leu48^{2.46}$ is located near the cytoplasmic end of TM2 and oriented towards the core of the receptor helix bundle close to the NPXXY motif in TM7, which is composed of the highly conserved residues $Asn^{7.49}$, $Pro^{7.50}$ and $Tyr^{7.53}$. $Leu^{2.46}$ has been described as being involved in a hydrophobic interaction with $Asn^{7.49}$ which may constrain the receptor in an inactive conformation[26], and was suggested to stabilise the ground state of rhodopsin[25]. The Q89A mutation was previously reported to increase the affinity of agonists and decrease the affinity for antagonists, perhaps through an indirect effect on the receptor[27], which we also observed here (Fig. 4). In the crystal structure of A_{2A}R bound to ZM241385, both T65A and A54L are located in TM2 facing the lipid bilayer.

The rationale for thermostabilising membrane proteins is to allow the use of short-chain detergents during both purification and crystallisation, which will improve the probability of success in well diffracting crystals that are suitable for structure determination[28]. Therefore, the success of any thermostabilisation procedure should be apparent during purification of the mutated receptor, because it should not aggregate during the procedure even if relatively harsh detergents are used.

Initial purification of A_{2A}R-GL26 showed that the receptor was present as both an unglycosylated and N-glycosylated product (results not shown), so the additional mutation N154A was introduced into GL26 to make the non-glycosylated mutant A_{2A}R-GL31. This was subsequently purified in NG and the product was monodisperse and pure, showing that the protein was ideal for crystallography. Indeed, crystals were obtained relatively easily and have been improved to diffract to better than 2.6 Å resolution; the structure of GL31 is currently under refinement. Thus the thermostabilisation strategy presented here for stabilising the agonist-bound form of GPCRs was successful and should be equally applicable to other GPCRs.

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CLAIMS

1. A mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

2. A mutant GPCR which when compared to the corresponding parent receptor, has a different amino acid (i) at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

3. A mutant GPCR according to Claim 1 or 2, wherein the mutant GPCR has increased stability of a particular conformation relative to its parent GPCR when residing in the same conformation.

4. A mutant GPCR according to Claim 3, wherein the mutant GPCR has increased stability in an agonist or antagonist conformation.

5. A mutant GPCR according to any of Claims 1-4, wherein the mutant GPCR has increased stability to any one or more of heat, a detergent, a chaotropic agent and an extreme of pH.

6. A mutant GPCR according to any of Claims 1-5, wherein the mutant GPCR is one which when compared to the corresponding parent receptor, has a different amino acid at positions that correspond to Leu 48, Gln 89, Thr 65 and Ala 54, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

7. A mutant GPCR according to any of Claims 1-6, wherein the parent GPCR is any of an adenosine receptor, a serotonin receptor, a β-adrenergic receptor, a neurotensin receptor, a muscarinic receptor, or an orexin receptor.

8. A mutant GPCR according to Claim 7, wherein the mutant GPCR is a mutant adenosine A_{2A}, A_{2B}, A_3 or A_1 receptor.

9. A mutant GPCR according to Claim 8, wherein the mutant adenosine receptor has an amino acid sequence which is at least 20% identical to that of the human adenosine A_{2A} receptor whose sequence is set out in Figure 7 (SEQ ID No: 1).

10. A method of producing a mutant GPCR with increased stability relative to its parent GPCR, the method comprising making one or more mutations in the amino acid sequence that defines a parent GPCR at a position corresponding to one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

11. A method of producing a mutant GPCR with increased stability relative to its parent GPCR, the method comprising making one or more mutations in the amino acid sequence that defines a parent GPCR (i) at a position which corresponds to any one or more of Ile 10, Val 12, Trp 29, Ser 47, Ala 50, Phe 62, Thr 65, Ala 72, Ala 73, Phe 79, Ile 80, Phe 83, Gln 89, Ala 97, Glu 212, Gln 214, Leu 216, Pro 217, Gly 218, Leu 225, Ala 236, Ile 238, Phe 242, Phe 258, and Val 282, and (ii) at a position which corresponds to any one or more of Leu 48, Ala 54, Val 57, Ser 90, Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Ala 231, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313 and Lys 315 according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

12. A mutant GPCR according to any of Claims 1-9, which has increased stability compared to its parent GPCR when exposed to a destabilising condition.

13. A composition comprising a mutant GPCR according to any of Claims 1-9, characterised in that the mutant GPCR is exposed to a destabilising condition effective to destabilise a parent GPCR to a greater extent than the mutant GPCR.

14. A mutant GPCR according Claim 12, or a composition according to Claim 13, wherein the mutant GPCR is membrane free.

15. A mutant GPCR according to Claim 12 or 14, or a composition according to Claim 13 or 14, wherein the mutant GPCR has increased stability compared to a parent GPCR in the absence of a ligand, or when in the presence of a ligand.

16. A mutant GPCR according to any of Claims 12, 14 and 15, or a composition according to any of Claims 13-15, wherein the mutant GPCR is in a non-aggregated form.

17. A mutant GPCR according to any of Claims 12 and 14-16, or a composition according to any of Claims 14-16, wherein the destabilising condition is any of heat, a detergent, a chaotropic agent, an extreme of pH, an organic solvent, an aqueous solution or a membrane free environment.

18. A mutant GPCR according to any of Claims 12 and 14-17, or a composition according to any of Claims 14-17, wherein the mutant GPCR has increased stability to any one of heat, a detergent, a chaotropic agent and an extreme of pH.

19. A mutant GPCR according to any of Claims 12 and 14-17, or a composition according to any of Claims 14-17, wherein the mutant GPCR has increased thermostability.

20. A mutant GPCR or composition according to Claim 19, wherein the mutant GPCR is at least 1°C more stable than its parent.

21. A mutant GPCR according to any of Claims 12 and 14-20, or a composition according to any of Claims 14-20, wherein the mutant GPCR is in a solubilised form.

22. A mutant GPCR according to any of Claims 12 and 14-21, or a composition according to any of Claims 14-21, wherein the mutant GPCR is substantially free of other proteins.

23. A mutant GPCR according to any of Claims 12 and 14-22, or a composition according to any of Claims 14-22, immobilized to a solid support.

24. A solid support to which is immobilized a mutant GPCR according to any of Claims 12 and 14-22, or a composition according to any of Claims 14-22.

25. Use of a mutant GPCR according to any of Claims 12 and 14-22, or a composition according to any of Claims 14-22, for crystallisation.

26. A mutant GPCR according to any of Claims 12 and 14-22, or a composition according to any of Claims 14-22, wherein the mutant GPCR is in a crystallised form.

27. A mutant GPCR or composition according to Claim 26, wherein the mutant GPCR is bound to an agonist or to an antagonist.

28. A mutant GPCR or composition according to Claim 26 or 27, wherein the mutant GPCR is one which when compared to the corresponding parent receptor, has a different amino acid at a position which corresponds to Leu 48, Gln 89, Thr 65, Ala 54 and Asn 154, according to the numbering of the human adenosine A_{2A} receptor as set out in Figure 7 (SEQ ID No: 1).

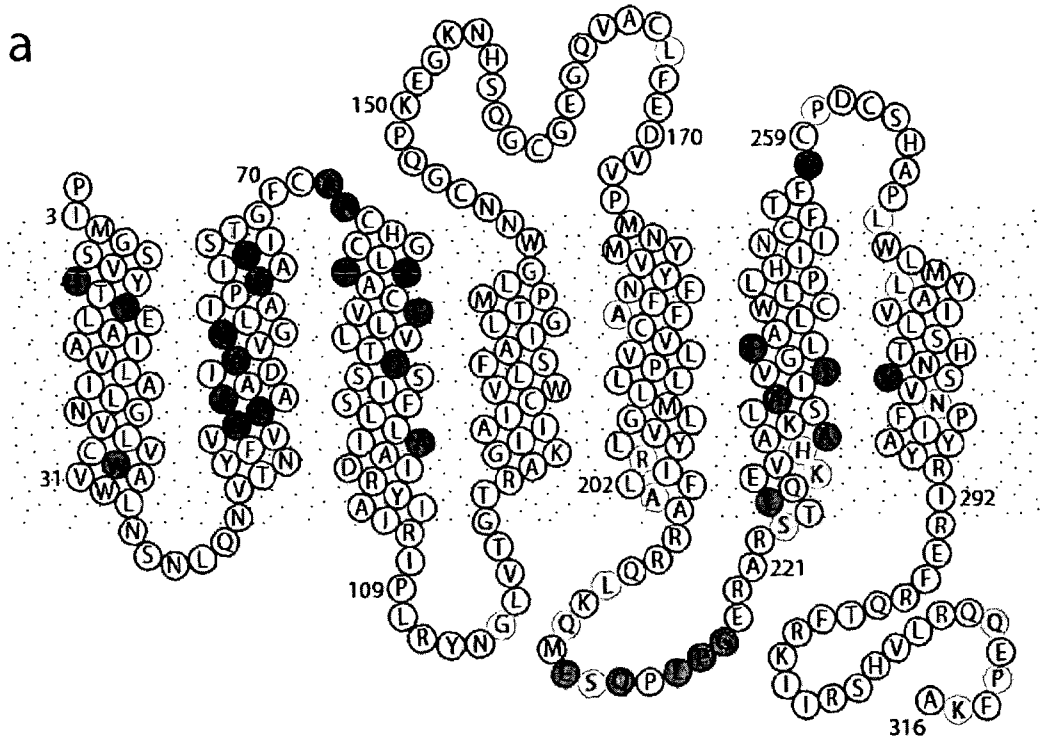
29. A mutant GPCR or composition according to Claim 28, wherein the mutant GPCR is bound to the agonist NECA.

30. Use of a mutant GPCR according to any of Claims 12 and 14-22, or a composition according to any of Claims 14-22 in drug discovery.

31. Use according to Claim 30 wherein the mutant GPCR or composition is used in a ligand binding screen or in assay development.

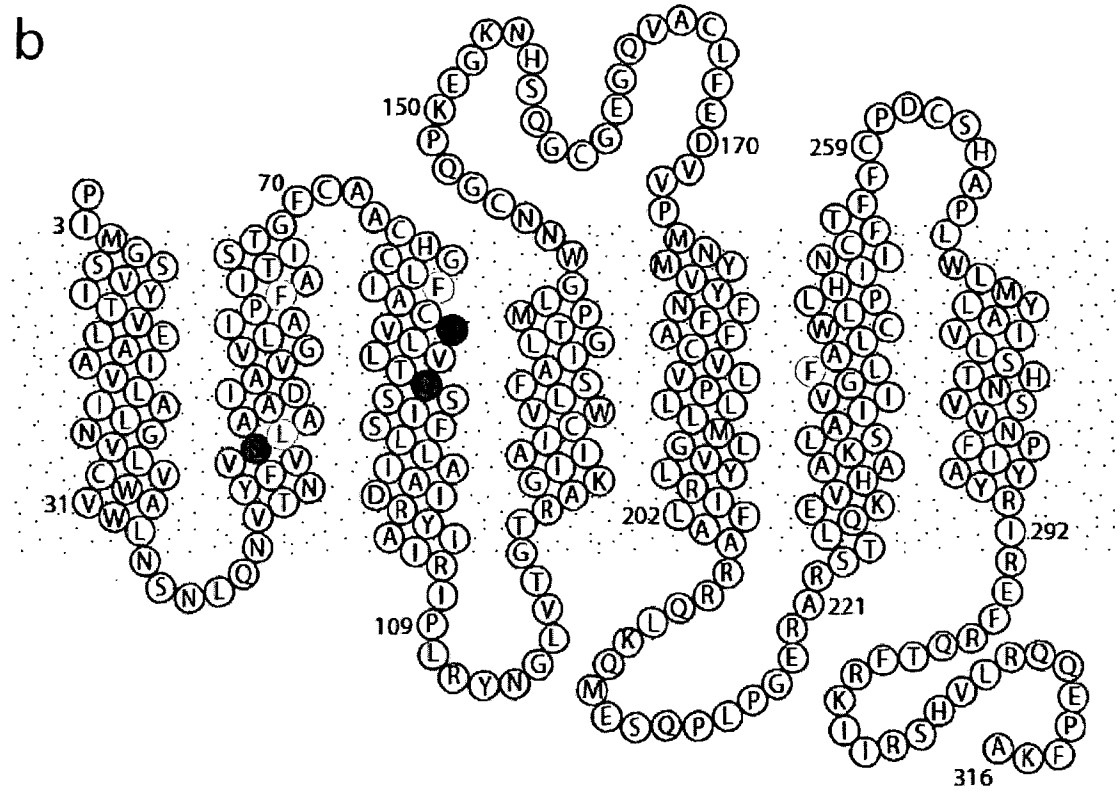
32. Use of a mutant GPCR according to any of Claims 12 and 14-22, or a composition according to any of Claims 14-22, as a biosensor.

FIGURE 1 (Page 1 of 2)



Thermostabilising mutations: I10, V12, W29, S47, L48, A50, A54, V57, F62, T65, A72, A73, F79, I80, F83, Q89, A97, E212, Q214, L216, P217, G218, L225, A231, A236, I238, F242, F258, V282
Mutations selected with unliganded receptor: G114, L167, A184, R199, A203, L208, Q210, S213, S223, K227, H230, P260, L267, L272, N284, Q311, P313, K315
Mutations selected with both assays: S90, G118, E219, R220, T224, Q226, L241, S263, T279

FIGURE 1 (Page 2 of 2)



Mutants that did not bind antagonist: L48, F62, F79, F242
Mutants less stable than wild type in antagonist conformation: S47, F83, Q89
Mutants more stable than wild type in antagonist conformation: V12, A50, A54, V57, T65, S90, A236, I238, V282

FIGURE 2

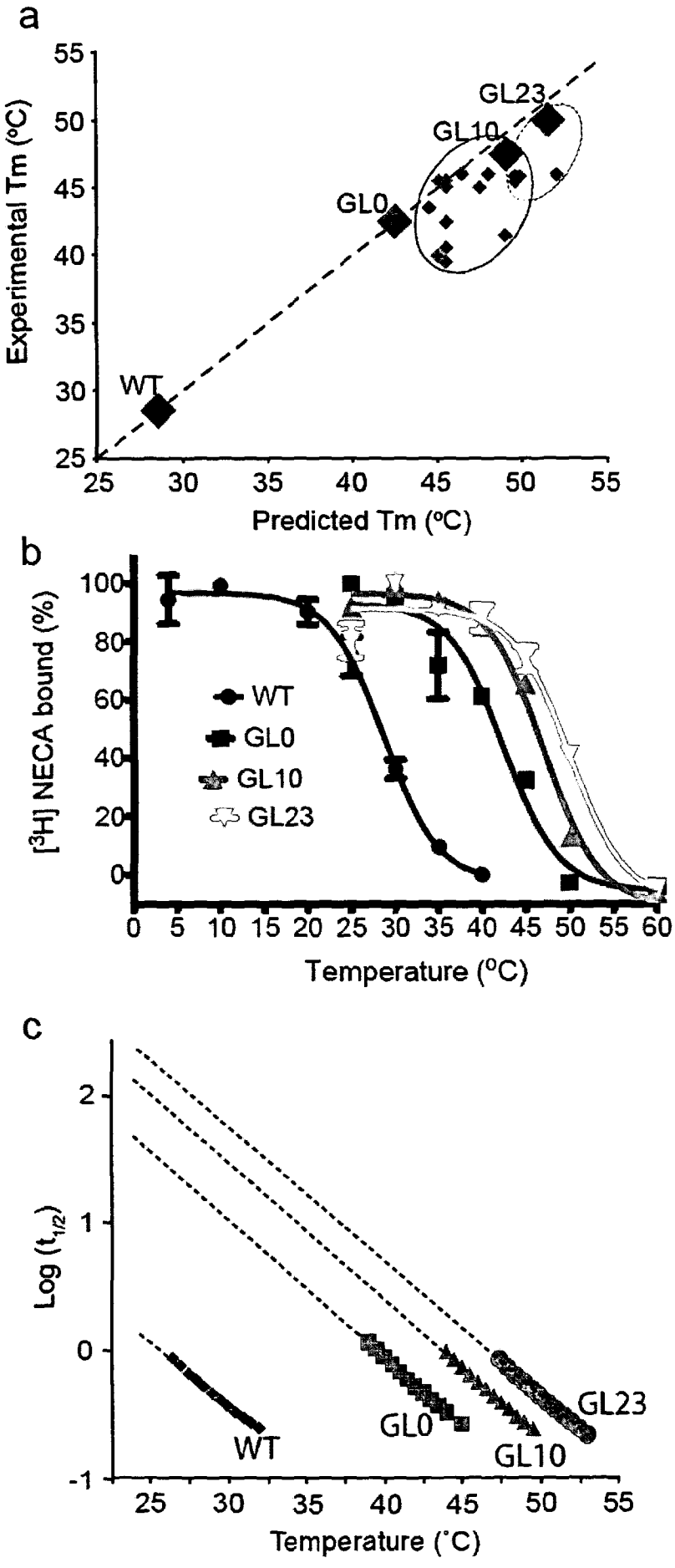

a
Experimental Tm (°C)
Predicted Tm (°C)
WT
GL0
GL10
GL23
b
[3H] NECA bound (%)
Temperature (°C)
WT
GL0
GL10
GL23
c
Log (t1/2)
Temperature (°C)
WT
GL0
GL10
GL23

FIGURE 3

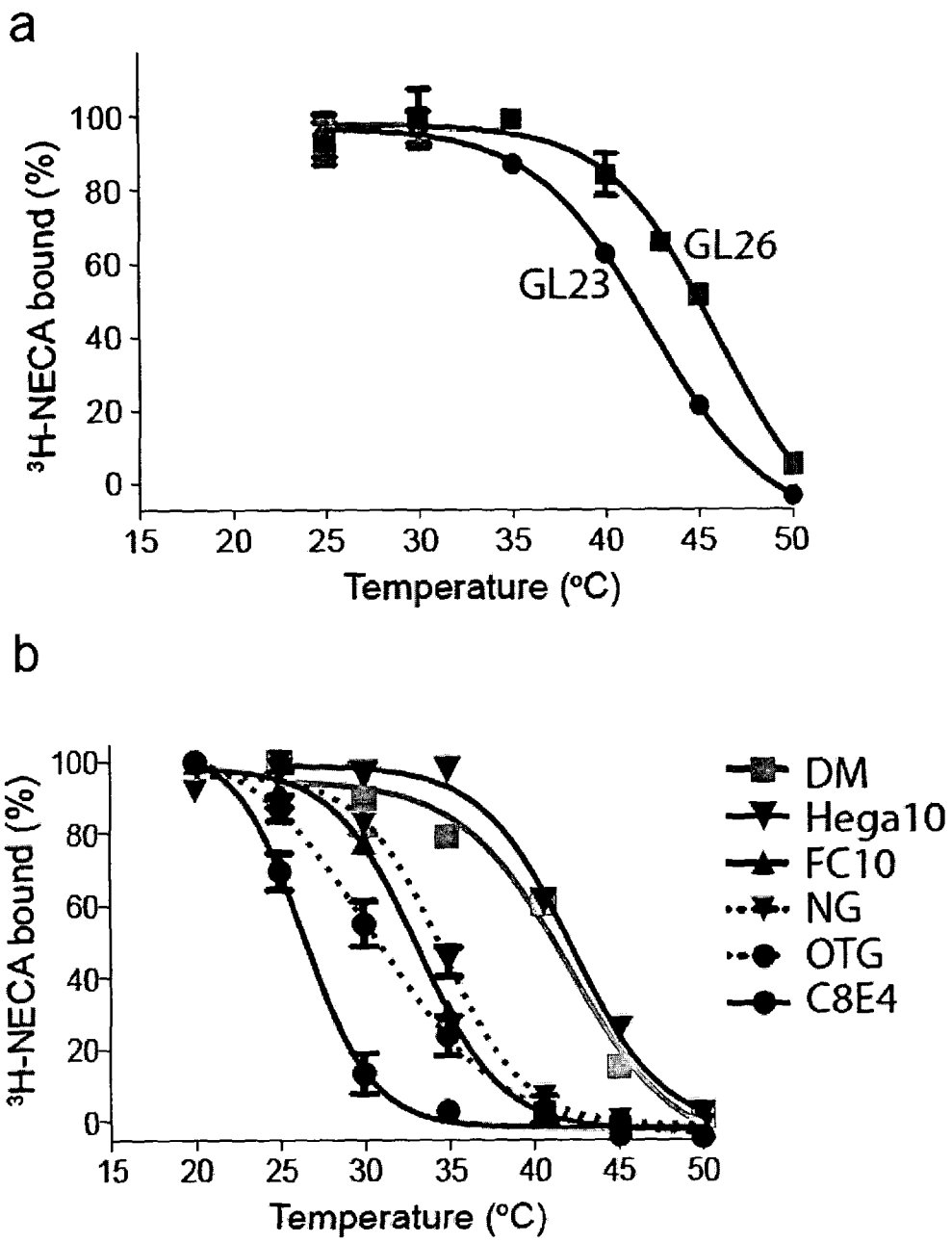
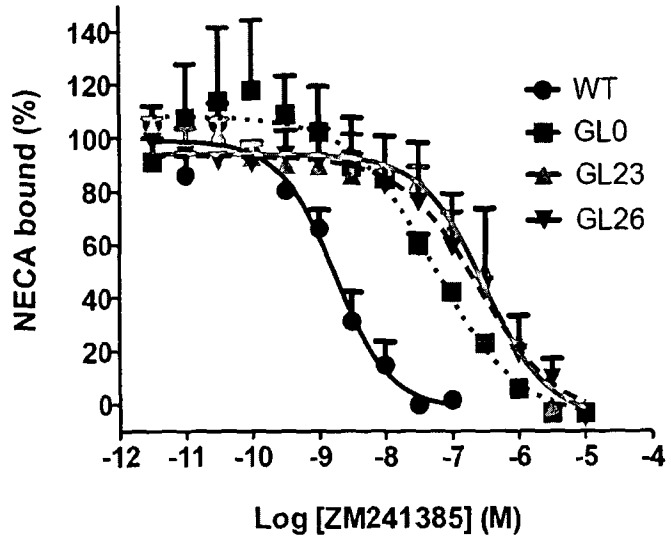
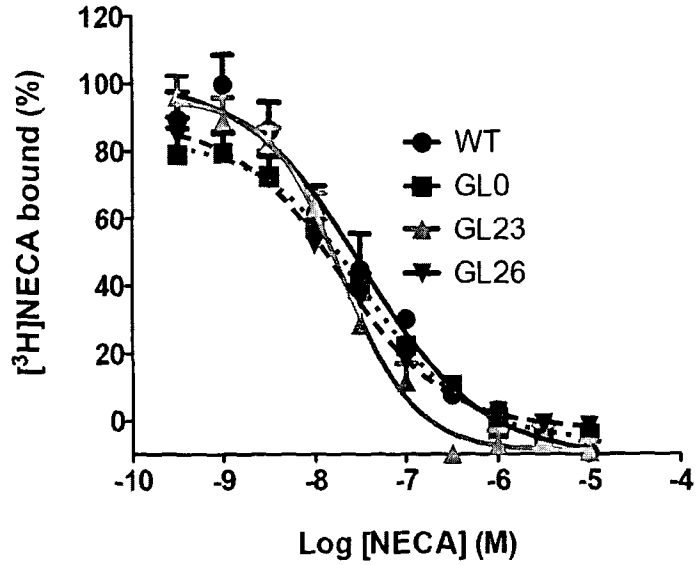

a
^{3}H-NECA bound (%)
100
80
60
40
20
0
15
20
25
30
35
40
45
50
Temperature (°C)
GL26
GL23
b
^{3}H-NECA bound (%)
Temperature (°C)
DM
Hega10
FC10
NG
OTG
C8E4

FIGURE 4

a


NECA bound (%)
140
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6 -5 -4
Log [ZM241385] (M)
WT
GL0
GL23
GL26

b


[3H]NECA bound (%)
120
100
80
60
40
20
0
-10 -9 -8 -7 -6 -5 -4
Log [NECA] (M)
WT
GL0
GL23
GL26

c

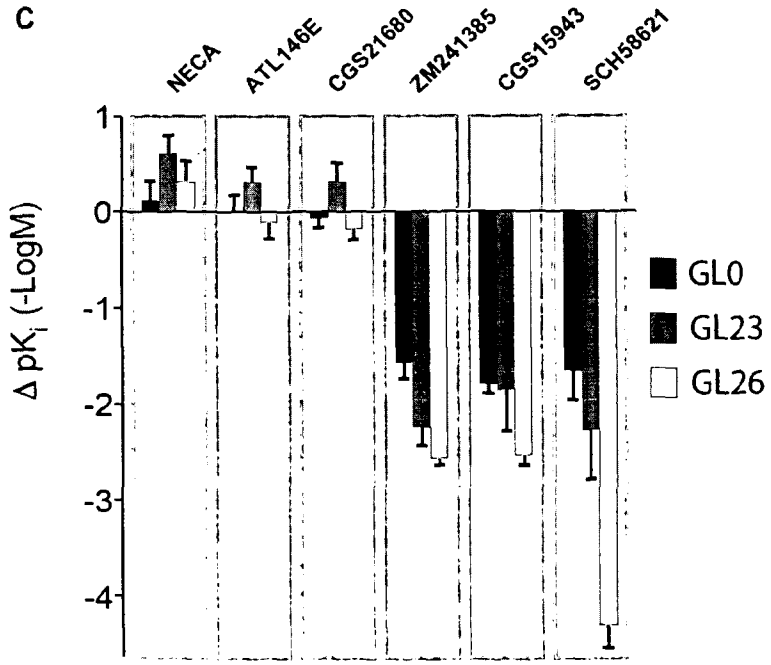

NECA
ATL146E
CGS21680
ZM241385
CGS15943
SCH58621
Δ pKi (-LogM)
1
0
-1
-2
-3
-4
GL0
GL23
GL26

FIGURE 5

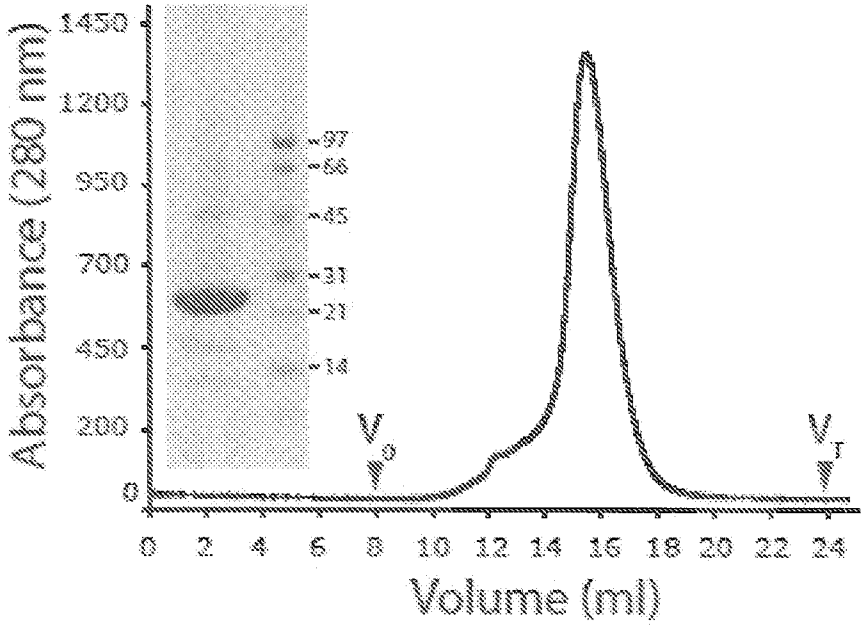

Absorbance (280 nm)
1450
1200
950
700
450
200
0
97
66
45
31
21
14
V_o
V_T
0 2 4 6 8 10 12 14 16 18 20 22 24
Volume (ml)

FIGURE 6

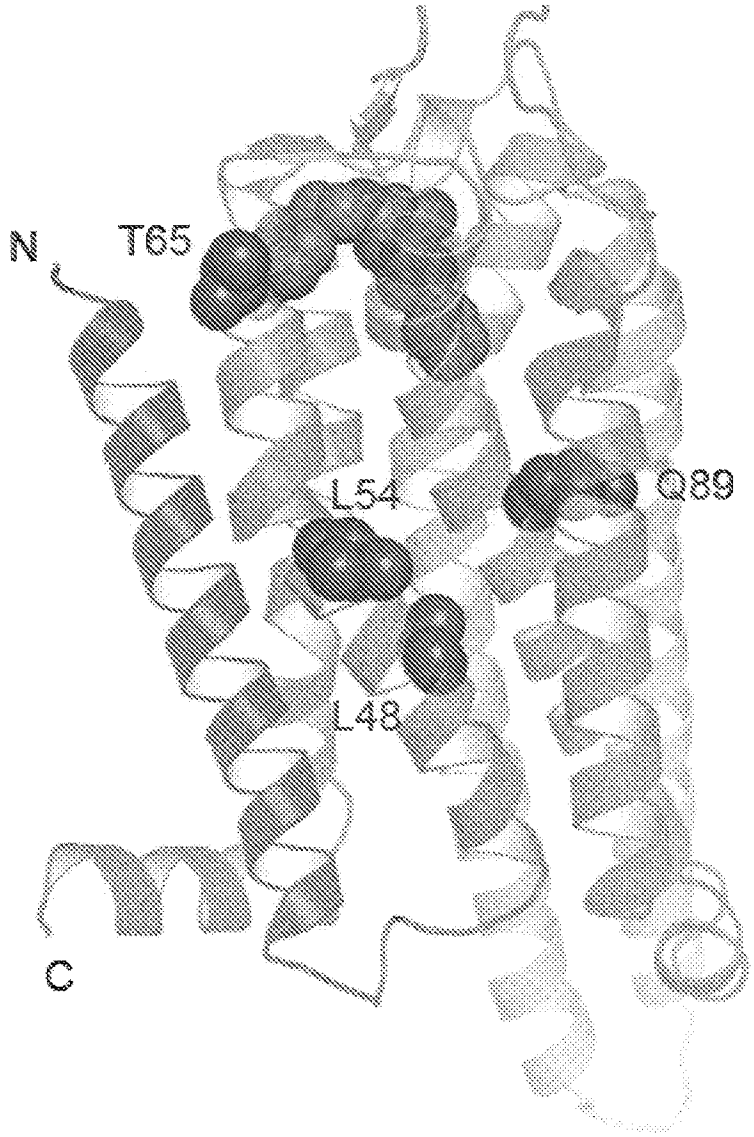


FIGURE 7

```
  1 MPIMGSSVYI TVELAIAVLA ILGNVLVCWA VWLNSNLQNV TNYFVVSLAA ADIAVGVLAI
 61 PFAITISTGF CAACHGCLFI ACFVLVLTQS SIFSLLAIAI DRYIAIRIPL RYNGLVTGTR
121 AKGIIAICWV LSFAIGLTPM LGWNNCGQPK EGKNHSQGCG EGQVACLFED VVPMNYMVYF
181 NFFACVLVPL LLMLGVYLRI FLAARRQLKQ MESQPLPGER ARSTLQKEVH AAKSLAIIVG
241 LFALCWLPLH IINCFTFFCP DCSHAPLWLM YLAIVLSHTN SVVNPFIYAY RIREFRQTFR
301 KIIRSHVLRQ QEPFKAAGTS ARVLAAHGSD GEQVSLRLNG HPPGVWANGS APHPERRPNG
361 YALGLVSGGS AQESQGNTGL PDVELLSHEL KGVCPEPPGL DDPLAQDGAG VS
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