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Abstract

G-protein coupled receptors (GPCRs) play a major role in cell-cell communication in the central nervous system. These proteins oscillate between various inactive and active conformations, the latter being stabilised by agonists. Although mutations can lead to constitutive activity, most of these destabilise inactive conformations, and none lock the receptor in an active state. Moreover, GPCRs are known to form dimers but the role of each protomer in the activation process remains unclear. Here we show that the heterodimeric GPCR for the main inhibitory neurotransmitter, the $GABA_B$ receptor, can be locked in its active state by introducing two cysteines expected to form a disulphide bridge to maintain the binding domain of the $GABA_{B1}$ subunit in a closed form. This constitutively active receptor cannot be inhibited by antagonists, but its normal functioning, activation by agonists and inhibition by antagonists, can be restored after reduction with dithiothreitol. These data show that the closed state of the binding domain of $GABA_{B1}$ is sufficient to turn ON this heterodimeric receptor and illustrate for the first time that a GPCR can be locked in an active conformation.

Introduction

G-protein coupled receptors (GPCRs) play a major role in cell-cell communication, especially in the brain where they are involved in the tuning of fast synaptic transmission. Several classes of GPCRs can be defined based on sequence similarity (Kolakowski, 1994; Bockaert and Pin, 1999; Fredriksson et al., 2003). GPCRs for the two main neurotransmitters, GABA and glutamate (the $GABA_B$ and metabotropic glutamate (mGlu) receptors) are part of the class-III GPCRs, together with those activated by Ca^{2+}, sweet molecules and some pheromones (Pin et al., 2003). These receptors form dimers, but the $GABA_B$ receptor is an obligatory heterodimer constituted of the homologous $GABA_{B1}$ and $GABA_{B2}$ subunits (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). $GABA_{B1}$ is responsible for GABA recognition (Galvez et al., 2000a; Kniazeff et al., 2002), whereas $GABA_{B2}$ is necessary for the correct trafficking of $GABA_{B1}$ to the cell surface (Margeta-Mitrovic et al., 2000; Pagano et al., 2001) and is involved in G-protein activation (Galvez et al., 2001; Margeta-Mitrovic et al., 2001b; Robbins et al., 2001; Duthey et al., 2002).

Ligand binding site of class-III GPCRs is located in a large extracellular domain (Takahashi et al., 1993) homologous to some periplasmic binding proteins (O'Hara et al., 1993) and to the binding domain of other receptors such as ionotropic glutamate, atrial natriuteric peptide (ANP) and some tyrosine kinase receptors (Felder et al., 1999; Vicogne et al., 2003). X-ray structures have been solved for the extracellular domains of mGlu1 (Kunishima et al., 2000; Tsuchiya et al., 2002) and ANP receptor types A and C (He et al., 2001; van den Akker, 2001) produced as soluble proteins. These domains are bilobate proteins often called Venus Flytrap modules (VFTMs) and share similar sequence homology with the extracellular domain of the $GABA_B$ subunits (Figure 1a). The mGlu1 VFTM was

observed either in an open or a closed conformation whereas that of ANPR is only found in a closed conformation.

Both mGlu1 and ANP VFTMs homodimerise at the level of their lobe-I, leaving the two lobes-II (connected to the membrane domain) far apart in the absence of agonists (Fig. 1b,c). In the presence of agonists, a change in conformation brings together lobes-II possibly forcing the membrane domains to interact differently (Fig. 1b,c). In the case of mGlu1, this results from: 1) closure of at least one VFTM, and 2) a change in the relative orientation of the VFTMs (Fig. 1b). In the case of the ANPR, this results from the binding of ANP between the two VFTMs (Fig. 1c). Whether the dimer of $GABA_B$ VFTMs functions like that of mGlu or ANP receptors remains unknown.

Here we show that the introduction of two cysteines expected to lock the $GABA_{B1}$ VFTM in a closed state by a disulphide bridge is sufficient to lock the entire receptor in an almost fully active state. This reveals a similar activation mechanism for mGlu and $GABA_B$ receptors, and represents the first example of a GPCR that has been locked in its active state. As such this study brings much information on the way the GPCRs for the main neurotransmitters, glutamate and GABA, are activated.

Materials and methods

Materials

GABA, baclofen and CGP64213 were gifts from Drs. W. Froestl and K. Kaupman (Novartis Pharma, Basel, Switzerland). [^{125}I]-CGP64213 was purchase from Anawa (Zurich Switzerland). Fetal bovine serum (FBS), culture media and other solutions used for cell culture were from GIBCO-BRL-Life Technologies, Inc. (Cergy Pontoise, France). [^{3}H]-myo-inositol (23.4 Ci/mol) was purchased from Perkin–Elmer Life Science (NEN) (Paris, France). All other reagents used were of molecular or analytical grade where appropriate.

Phylogenetic analysis

Sequence alignment of the VFTMs of crystallised amide binding protein AmiC (pdb: 1pea), Leucine/isoleucine/valine binding protein (LIVBP, pdb: 2liv), natriuretic peptide receptor A and C (NPRA and NPRC, pdb: 1dp4 and 1jdn, respectively), and metabotropic glutamate receptor type 1 (mGlu1, pdb: 1ewk) was deduced after superimposition of their structures using SwissPdbViewer (v3.7) (Guex and Peitsch, 1997). Sequences of VFTMs of the rat NR2A subunit of the NMDA receptor, $GABA_{B1}$ and $GABA_{B2}$ subunits (GB1 and GB2), and tyrosine kinase receptor type 1 (RTK1) from Schistosoma mansoni were aligned on the structural multiple aligment according to (Paoletti et al., 2000), (Kniazeff et al., 2002), and (Vicogne et al., 2003), respectively. The phylogenetic tree was then constructed using the neighbour joining method (Saitou and Nei, 1987) with the command interface of the Clustal W 1.60 program (Thompson et al., 1994) using the default parameters and not excluding gaps. Bootstrap values were calculated using 1000 trials with seeds of 111. The tree was drawn using TreeView (v1.6.2) (Page, 1996).

Molecular modelling

Three-dimensional models of open and closed form of $GABA_{B1}$ VFTM were built as previously described (Kniazeff et al., 2002) using Modeller 6.0 alpha (Sali and Blundell, 1993). *In silico* mutagenesis and disulphide bridges modelling were performed using SwissPdbViewer program (v3.7) (Guex and Peitsch, 1997) and software default parameters. The figures were prepared using SwissPdbViewer.

Plasmids and site-directed mutagenesis:

The plasmids encoding the wild-type $GABA_{B1a}$ and $GABA_{B2}$ subunits epitope tagged at their N-terminal ends (pRK-$GABA_{B1a}$-HA and pRK-$GABA_{B2}$-cMyc), under the control of a CMV promoter, were described previously (Galvez et al., 2001; Pagano et al., 2001).

Mutant subunits, carrying single or multiple mutations, were obtain using the Quick-Change strategy (Stratagene, La Jolla, CA). Briefly, for each mutagenesis, two complementary 27-mers primers (Eurogentec, Bruxelles, Belgium) were designed to contain the desired mutation. To allow a rapid screening of mutated clones, primers carried an additional silent mutation introducing a new restriction site. The presence of the desired mutations and the absence of additional ones were confirmed by DNA sequencing (Genaxis, Nîmes, France). For double mutants two Quick-Change reactions were performed successively.

Cell culture and expression in HEK 293

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and transfected by electroporation as described elsewhere (Brabet et al., 1998; Franek et al., 1999). Unless stated otherwise, 10.10^6 cells were transfected with plasmid DNA containing HA-tagged $GABA_{B1}$ (2µg), cMyc-tagged $GABA_{B2}$

(2µg), and completed to total amount of 10 µg plasmid DNA with pRK_6. For determination of inositol phosphate accumulation, the cells were also tranfected with the chimeric Gαqi9 G-protein which allows the coupling of the recombinant heteromeric $GABA_B$ receptor to PLC (Franek et al., 1999).

Determination of inositol phosphate accumulation

Determination of inositol phosphate (IP) accumulation in transfected cells was performed in 96-wells plates ($0.2.10^6$ cells/well) after over-night labeling with ^{3}H-myo-inositols (0.5µCi/well) as described by others (Chengalvala et al., 1999) with some modifications. The stimulation was conducted for 30 minutes in a medium containing 10mM LiCl and the indicated concentration of agonist or antagonist. The reaction was stopped by replacing the medium by 0.1M formic acid. Supernatants were recovered and IP were purified by ion exchange chromatography using DOWEX AG1-X8 resin (Biorad, Marnes-la-Coquette, France) in 96 well filter plates (ref: MAHVN4550 Millipore, Bedford, MA). Total radioactivity remaining in the membrane fractions was counted after treatment of cells with a solution containing 10% triton X-100 and 0.1N NaOH. Radioactivity was quantified using Wallac 1450 MicroBeta liquid scintillation counter. Data were expressed as the amount of total IPs produced over the amount of radioactivity remaining in the membranes plus the produced IP. Unless stated otherwise, all data are means ± sem of at least 3 independent experiments and expressed in percentage of wild-type activity after 30min application of 1mM GABA. The dose-response curves were fitted using the GraphPad Prism program and the following equation "$y=[(y_{max}-y_{min})/(1+(x/EC_{50})^{nH}))+y_{min}$" where the EC_{50} is the concentration of the compound necessary to obtain 50% of the maximal effect and nH is the Hill coefficient.

Ligand binding assay

Ligand binding assay on intact HEK 293 cells was performed as previously described using 0.1 nM [^{125}I]-CGP64213 (Galvez et al., 2001). Displacement curves were performed with at least 7 different concentrations of the displacer and the curves were fitted according to the equation: "$y=[(y_{max}-y_{min})/(1+(x/IC_{50})^{nH}))+y_{min}$" where the IC_{50} is the concentration of the compound that inhibits 50% of bound radioligand and nH is the Hill coefficient. Ki values were calculated according to the equation $IC_{50}=Ki(1+[RL]/Kd)$, where [RL] and Kd are the concentration and dissociation constant of the radioligand. Kd was determined assuming Ki=Kd in the case of CGP64213.

Western Blotting

Western blotting was performed as previously described (Kniazeff et al., 2002) using the rabbit polyclonal anti-HA antibody (Zymed, San Francisco, CA) and the anti-rabbit HRP antibody (Amersham, Saclay, France). The signal was revealed using an ECL chemioluminescent assay.

Anti HA ELISA assay for quantification of cell surface expression

Twenty four hours after transfection (10.10^6 cells, HA-tagged $GABA_{B1}$ (2µg) and cMyc-tagged $GABA_{B2}$ (2µg) subunits), cells were fixed with 4% paraformaldehyde and then blocked with PBS + 5% FBS. After 30 minutes reaction with primary antibody (monoclonal anti-HA clone 3F10 (Roche, Basel, Switzerland) at 0.5µg/mL) in the same buffer, the goat Anti-Rat antibody coupled to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA) was applied for 30 minutes at 1µg/mL. After intense washes with PBS, secondary antibody was detected and quantified instantaneously by chemiluminescence using

Supersignal® ELISA femto maximum sensitivity substrate (Pierce, Rockford, IL) and a Wallac Victor^2 luminescence counter.

GTP-γ-^{35}S binding measurements

Cells were transfected using PolyFect transfection reagent (Qiagen, Hilden, Germany) under oprimized conditions. Complex were formed using total amount of 8µg plasmid DNA with 60µL of polyfect in 300µL of serum free antibiotic free DMEM for 10 minutes and then added to cells at 40-60% confluency. According expression results, the amount of DNA is $GABA_{B1}$ 2µg, $GABA_{B2}$ 1µg, Gαo1c 2µg and pRK_6 3µg for wild-type receptor and $GABA_{B1}$ 2µg, $GABA_{B2}$ 2µg, Gαo1c 2µg and pRK_6 2µg for CC1 and CC2 mutants.

Forty-eight hours after transfection, cells were scraped in lysis buffer (15mM Tris, 2mM MgCl2, 0.3mM EDTA pH 7.4) and centrifuged twice. The pellet was resuspended in Tris (50mM), MgCl2 (3mM), EGTA (0.2mM), NaCl (60mM) pH 7.4 using a potter. The GTP-γ-[^{35}S] (Amersham, Little Chalfont, UK, 1099 Ci/mMol) binding was performed in 96-wells filtration plates (ref: MAFCN0B50, Millipore, Bedford, MA) equilibrated with Tris.(50mM) MgCl2 (5mM) pH7.4. Membranes (5µg per well) were pre-incubated or not with GABA (15 minutes, 1 mM, final volume 20µL). The plate was incubated one hour at 30°C after addition of 60µL of incubation buffer (50mM Tris, 1mM EDTA, 10µM GDP, 5mM MgCl2, 0.01mg/mL leupeptine, 100mM NaCl, and 0.4nM GTP-γ-[^{35}S]) and 20µL of H_2O. After vacuum filtration, plate filter washing (3 times with 250µL Tris 50mM) and drying, the radioactivity was measured using a Wallac 1450 MicroBeta liquid scintillation counter.

Results

Double cysteine mutations expected to lock the $GABA_{B1}$ VFTM in a closed state generate constitutively active receptors

Based on 3D models of the open and closed forms of the $GABA_{B1}$ VFTM (Galvez et al., 1999; Galvez et al., 2000a; Kniazeff et al., 2002), some positions were selected to introduce cysteines that could form a disulphide bridge in the closed state only. As depicted in Fig. 2, the side chains of Ser247 or Ser246 from lobe-I and those of Thr315 or Glu316 from lobe-II are far apart in a model of the open form (Fig. 2a), but are in close proximity in the closed form (Fig. 2b,c). Indeed, in the double mutants S247C-T315C (CC1) (Fig. 2d) and S246C-E316C (CC2) (data not shown), the distance between the sulphur atoms in the closed form models is compatible with the formation of a disulphide bond (1.9 and 2.1 Å for CC1 and CC2, respectively). Double cysteine mutations were introduced in $GABA_{B1}$ and the CC1 and CC2 mutants were analysed for function after co-expression with wild-type $GABA_{B2}$. Expression of the CC mutants in HEK293 cells lead to a high constitutive activity in the absence of agonist reaching 69.8 ± 4.8 (CC1) and 48.8 ± 4.3 (CC2) % ($n>3$) of the maximal GABA-induced activity measured with the wild-type receptor (Fig. 3a). In contrast, no significant increase in constitutive activity can be detected with any of the single cysteine mutants (Fig. 3b). Such a property of the CC mutants can be detected using either the artificial coupling of the $GABA_B$ receptor to phospholipase C with the chimeric Gqi9 protein (Fig. 3a), but also through the direct activation of Go protein as shown by GTPγ[^{35}S] binding (Fig. 3c).

A disulphide bridge is involved in the constitutive activity of CC mutants

In agreement with the involvement of a disulphide bond in the high constitutive activity of CC mutants, this activity can no longer be detected after reduction with DTT (Fig.

3d). This later effect does not result from a destabilisation of any of the subunits due to reduction of native disulphide bonds for two reasons. First, DTT treatment of cells expressing the wild-type receptor had no significant effect on the basal activity and only a small decrease in GABA-mediated response was observed (Fig. 3d). Second, after reduction, CC mutants can still be activated by agonists (Fig. 3d) with an affinity similar to that measured on the wild-type receptor (Table 1).

The constitutive activity of CC mutants is not inhibited by competitive antagonists

Competitive antagonists of mGluRs have been shown to bind in the open form of the VFTM, as illustrated with the mGlu1 (Tsuchiya et al., 2002) and mGlu8 (Bessis et al., 2002) receptors. Accordingly, a receptor locked in a closed state would not be expected to bind competitive antagonists. As shown in Fig. 4a, the antagonist CGP64213 did not inhibit the basal activity of the CC mutants whereas it was able i) to inhibit the basal activity of the wild-type receptor, ii) to antagonise GABA-mediated responses of single cysteine mutants (Table 1). Moreover, after DTT treatment, this antagonist become effective in fully inhibiting GABA activation of the CC mutants (Fig. 4b) demonstrating that a disulphide bridge, but not the presence of the two cysteines, is responsible for the absence of antagonist action. These data show that the constitutively active receptor is locked in its active state and cannot return to the inactive antagonist-stabilised state.

Not all surface expressed CC1 mutants are locked in an active state

Although high constitutive activity can be observed in cells expressing the CC1 mutant, GABA (as well as its chlorophenyl derivative baclofen (data not shown)) could still further increase the response with a potency identical to that measured on the wild-type receptor (Fig. 5a). This could result from the disulphide bridge-stabilised receptors being in a

partially active conformation. However, the agonist-induced response can be fully antagonised by CGP64213 with the same potency as that measured on the wild-type receptor (Fig. 5b), indicating that the antagonist has access to agonist-activated receptors. These data suggest that there are two populations of receptors, some constitutively active and insensitive to antagonists, and some that can be activated by agonists and inhibited by CGP64213. In agreement with this proposal, a small amount of specific [^{125}I]-CGP64213 binding can be detected in cells expressing the CC1 mutant, and this binding was largely increased after DTT treatment (about 5 fold) in contrast to the wild-type receptor (Fig. 6a). This effect was not due to a change in the CGP64213 affinity since the same Ki values for this antagonist could be determined by displacement studies on CC1 expressing cells before and after DTT-treatment (Table 1). This shows that reduction reveals new binding sites in cells expressing the CC1 mutant. Finally, the specific binding of [^{125}I]-CGP64213 after DTT treatment represented half of that determined on cells expressing the wild-type receptor, in agreement with a two fold lower expression level of this receptor (Fig. 6b,c). Therefore, most CGP64213 binding sites have been unmasked after DTT reduction. Taken together, these data show that 75.5 ± 6.9 % of the binding sites in cells expressing the CC1 mutant are inaccessible to CGP64213 due to a disulphide bridge. Thus the effect of GABA on these cells likely results from the 25% remaining receptors since this effect is fully inhibited by this antagonist. Another conclusion from these data is that GABA is unlikely able to activate the constitutively active, CGP64213-insensitive form of the receptor.

We then examined whether the proportion of disulphide-locked receptors could be modulated by oxidising agents DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) or Cu-Phenantroline, by agonists that are expected to stabilise the closed state, or by antagonists expected to prevent the formation of the closed state. None of these treatments alone or in combination lead to a modification of constitutive activity (Fig. 7). Moreover, after DTT

treatment, constitutive activity of the CC1 mutant could not be restored after 30 min exposure to the oxidising agents (data not shown). This suggests that all disulphide-locked receptors are formed inside the cells rather than after plasma membrane insertion.

Constitutive activity of the disulphide-locked CC1 mutant is close to the agonist-induced activity of the wild-type receptor.

We then examined whether the constitutive activity of the CC1 mutant corresponds to a fully active form of the receptor. Surface expression was determined with an ELISA assay, and second messenger formation was measured in cells expressing various amount of the $GABA_B$ heterodimer. A saturating curve was observed and the slope at the origin of the curves is indicative of the specific activity of the receptor (amount of second messengers produced per receptor) (Fig. 8). In absence of agonist, the specific activity of CC1 is about half the specific activity of the wild-type receptor activated by GABA ($0.7x10^{-3}$ and $1.4x10^{-3}$ arbitrary units, respectively) but, after DTT treatment, this activity is decreased to the level of the basal activity of the wild-type receptor. Since only 75% of the CC1 receptors are expected to be stabilised in an active form by a disulphide bridge (see above), this indicates that the specific activity of the constitutively active form of CC1 receptors is close to the specific activity of the agonist-activated wild-type receptors ($1.0x10^{-3}$ and $1.4x10^{-3}$ arbitrary units respectively).

The curves obtained clearly saturate indicating a maximal IP formation could be measured with a non saturating amount of receptors expressed at the cell surface. This is in contrast with what can be observed under similar condition with the Gq-coupled mGlu5 receptor (Goudet et al., manuscript in preparation). This may therefore results from the use of the chimeric G-protein α subunit Gqi9 co-transfected with the receptor to make it able to

activate PLC. It is possible that either the amount of Gqi9, or endogenous βγ subunits is limiting when the receptor is over-expressed.

Double cysteine mutations in the $GABA_{B2}$ VFTM does not influence receptor function

Can the closure of the $GABA_{B2}$ VFTM also lead to receptor activation? To test this possibility cysteines were introduced in the $GABA_{B2}$ VFTM at the same positions as in $GABA_{B1}$ but neither constitutive activity nor changes in agonist efficacy and potency were detected (data not shown). In order to examine whether such cysteines could form a disulphide bond in the $GABA_{B2}$ VFTM, and as such to lock this domain in a closed state, wertern blots experiments were conducted under reducing and non reducing (DTT 100 mM, pH 8) conditions. Such an experiment performed with the full length receptor did not lead to a clear conclusion likely because of the large size of these receptor subunits. As such, constructs were created (ΔGB1 and ΔGB2) in which a stop codon was introduced after TM1 leading to the expression of the VFTM attached to the membrane by the first transmembrane domain. As shown in Fig. 9, these constructs migrated faster under non-reducing condition, in agreement with the proposed existence of native disulphide bridges in the VFTM of both $GABA_{B1}$ and $GABA_{B2}$ (Galvez et al., 2000a; Kniazeff et al., 2002). Of interest, both ΔGB1-CC and ΔGB2-CC migrated slightly faster than the wild-types under non-reducing condition, consistent with the existence of an additional disulphide bridge in these constructs.

Discussion

The present study indicates that the introduction of two cysteines expected to lock the $GABA_{B1}$ VFTM in a closed form generates a constitutively active heterodimeric $GABA_B$ receptor.

A disulphide bond locks the $GABA_B$ receptor in an active state.

GPCRs are assumed to oscillate between various inactive (R) and active (R*) conformations (Lefkowitz, 1993; Samama et al., 1993; Leff, 1995; Farrens et al., 1996; Christopoulos and Kenakin, 2002), the latter being stabilised by agonists, whereas the former is stabilised by inverse agonists. Mutations have been identified that lead to constitutive activity (Lefkowitz, 1993; Samama et al., 1993; Leff, 1995; Christopoulos and Kenakin, 2002; Parnot et al., 2002), but these have been shown to destabilise the inactive conformations rather than to stabilise an active one (Parnot et al., 2002). As such these mutations are expected to displace the R<->R* equilibrium towards R*, as demonstrated by the inhibition of constitutive activity by inverse agonists, but did not bring much information on the active conformation of the receptor. The $GABA_{B1}$ CC mutants appear to be locked in an active state through the formation of an additional disulphide bond. Indeed, the R<->R* equilibrium appears to no longer exist since the receptor cannot return to the inactive state even in the presence of high concentration of the inverse agonist CGP64213. Only DTT treatment allowed the receptor to return to an inactive state. This represents the first example of a constitutively active form of a GPCR resulting from the locking of an active state.

A disulphide bond locks the $GABA_{B1}$ VFTM in a closed state.

Several data are consistent with the two cysteines introduced in the CC1 and CC2 mutants involved in a disulphide bridge that locks the $GABA_{B1}$ VFTM in a closed state. First of all, the VFTM of the CC1 mutant migrates faster than the wild-type in acrylamide gels under non-reducing conditions. Secondly, the distance between the two sulphydryl groups introduced is consistent with the formation of a disulphide bond in a closed form model of the $GABA_{B1}$ VFTM only. Thirdly, the $GABA_B$ antagonist CGP64213 is unable i) to inhibit the constitutive activity of the CC mutants and ii) to bind on the disulphide-locked receptor although this antagonist can bind and exert its normal antagonist action after DTT treatment. Indeed, as observed for the mGlu1 (Tsuchiya et al., 2002) and mGlu8 (Bessis et al., 2002) receptors, many $GABA_B$ competitive antagonists are larger than agonists and cannot fit into a closed form model of the binding site. Finally, modelling and mutagenesis studies suggested that the active form of the $GABA_{B1}$ VFTM corresponds to a closed form since residues from both lobes are involved in agonist binding (Galvez et al., 1999; Galvez et al., 2000a; Bernard et al., 2001; Costantino et al., 2001; Kniazeff et al., 2002).

However, the absence of modulation by oxidants or ligands expected to help or prevent the formation of the disulphide bridge was surprising. Accordingly, the disulphide-locked receptors are likely formed during the synthesis of the receptor in the endoplasmic reticulum. When the receptor is correctly folded and at the cell surface, in the absence of the pre-formed disulphide bond or after its reduction with DTT, the sulphydryl groups may not be in a correct orientation to spontaneously form an disulphide bond. Indeed, in our 3D model, the side chains of mutated residues had to be manually reoriented for the disulphide bridge to form. Moreover, this area in the $GABA_{B1}$ VFTM has been proposed to constitute a Ca^{2+}-binding site (Galvez et al., 2000b; Costantino et al., 2001), therefore stabilising a precise position of the side chains of the surrounding residues and possibly preventing the formation of a disulphide bond.

The disulphide-locked active state is close to the agonist-stabilized active state.

Although cells expressing the CC1 or CC2 mutants display a high constitutive PLC activity, IP formation could still be increased by $GABA_B$ agonists. This may appear surprising in the case of the CC2 mutant since Ser246 (mutated into Cys in this mutant) has previously been proposed to form a H-bond with the carboxylic function of $GABA_B$ ligands (Galvez et al., 2000a; Bernard et al., 2001; Costantino et al., 2001; Kniazeff et al., 2002). However, since an SH group can also form H-bonds, it is likely that the replacement of the OH group of Ser246 by a SH group has minor effect on ligand binding affinities.

In the case of the ionotropic glutamate receptor subtypes, partial agonists stabilised a partially closed form of the glutamate binding domain (Armstrong and Gouaux, 2000; Armstrong et al., 2003). Therefore, one possibility to explain the agonist-induced response in CC-mutant expressing cells was that the disulphide bond locked the receptor in a partially closed form, that could be further closed in the presence of agonist. However, our data indicate that this is unlikely the case but rather that the agonist-mediated response is due to a certain fraction of the receptors at the cell surface that are not locked in their active state by a disulphide bridge. Indeed, a fraction of the CC mutants can bind with a normal affinity the antagonist CGP64213, and the agonist-induced activity can be inhibited by the antagonist with a wild-type Ki. Taken this into consideration, together with the receptor density at the cell surface, we estimated that the specific activity of the constitutively active disulphide-locked CC1 mutant is close to that of the agonist-stabilised form of the wild-type receptor. This suggests that the conformation of the disulphide-locked CC1 VFTM is close to that of the agonist bound form of the wild-type receptor.

Comparison with mGlu1 and ANP receptors.

As shown in Fig. 1a, the $GABA_B$ VFTMs are as distant from the mGlu1 VFTM than from any other VFTMs. Moreover, the large insertions found in mGlu VFTMs and conserved in the related Ca^{2+}-sensing, pheromone and taste receptors, are not found in the $GABA_B$ VFTMs. Finally, whereas the mGlu-like receptors possess a cysteine-rich domain that connects their VFTM to the heptahelical domain, the $GABA_B$ subunits do not. In contrast, the $GABA_B$ VFTMs share a significant higher similarity with the VFTM of the monotopic receptor RTK1 (Vicogne et al., 2003), leaving open the possibility that the dimer of VFTMs of the dimeric $GABA_B$ receptor functions like those of dimeric monotopic receptors. However, in the case of the monotopic receptor for the natriuretic peptide, both VFTMs are in a closed conformation even in the inactive state (He et al., 2001; van den Akker, 2001) (Fig. 1c), whereas agonists appear to stabilise a closed form of the VFTM in the case of the mGlu receptors (Kunishima et al., 2000; Bessis et al., 2002; Tsuchiya et al., 2002) (Fig. 1b). Accordingly, our data suggest the dimer of $GABA_B$ VFTMs functions like that of mGlu receptors despite some structural dissimilarities between these two types of receptors.

Role of $GABA_{B2}$ VFTM in $GABA_B$ receptor activation.

$GABA_{B2}$ possesses a VFTM similar to that of $GABA_{B1}$. Although required for $GABA_B$ receptor activation (Galvez et al., 2001; Margeta-Mitrovic et al., 2001a), the $GABA_{B2}$ VFTM does not appear to bind any ligand (Kniazeff et al., 2002). Our data show that introduction of two cysteines at the same position as in CC1 and CC2 mutants in the $GABA_{B2}$ VFTM does not change the properties of the heteromeric $GABA_B$ receptor. However, as observed with the $GABA_{B1}$ CC mutant, the $GABA_{B2}$ CC migrates faster than the wild-type under non reducing condition, consistent with the existence of an additional disulphide bridge in this mutant. This suggests that the closure of $GABA_{B2}$ is not sufficient to activate the receptor. Indeed, either $GABA_{B2}$ VFTM is always closed or the closure of

$GABA_{B2}$ VFTM has no detectable effect on the function of the receptor. This is consistent with the closure of $GABA_{B1}$ VFTM being sufficient for $GABA_B$ receptor activation, in agreement with our proposal that $GABA_B$ agonists bind in the $GABA_{B1}$ VFTM only (Kniazeff et al., 2002). Moreover, our data also suggest that even an artificial ligand interacting in the cleft of $GABA_{B2}$ is not expected to have an important effect on the functioning of the heterodimer. .

Allostery between the 4 main domains of the heterodimeric $GABA_B$ receptor.

Although GABA binds to the $GABA_{B1}$ VFTM only, the $GABA_{B2}$ 7TM is critical for G-protein coupling (Galvez et al., 2001; Margeta-Mitrovic et al., 2001b; Robbins et al., 2001; Duthey et al., 2002). How can the closure of the $GABA_{B1}$ VFTM lead to the change in conformation of the $GABA_{B2}$ 7TM domain necessary for G-protein activation? In the case of mGlu receptors, it has been proposed that the change in the relative orientation of the two VFTMs, observed upon glutamate binding, may be required for activation (Kunishima et al., 2000; Jensen et al., 2001; Jensen et al., 2002). Based on this hypothesis, the closure of the $GABA_{B1}$ VFTM possibly leads to a change in the relative orientation of the two VFTMs in the heterodimer as proposed for the mGlu1 receptor, no matter whether the $GABA_{B2}$ VFTM is in a closed or open conformation (Fig. 10).

Conclusion

Our present study brings much information on how GABA activates the $GABA_B$ receptor, and on the specific roles played by each subunit in this heteromeric receptor. Moreover, such information may be of interest for the understanding of the activation process of many other GPCRs, Indeed, the rhodopsin-like class-I GPCRs also form dimers (Bouvier, 2001; Fotiadis et al., 2003). It is still unknown whether such dimers are required for activation

and whether, in such a case, agonist occupation of a single protomer is sufficient for full activation of the receptor. Taken into account our data and that retina can detect a single photon even though rhodopsin forms oligomers of dimers (Fotiadis et al., 2003; Liang et al., 2003), one may propose that an initial change in conformation in a single subunit may be sufficient to trigger the activation of some dimeric GPCRs.

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Legends to figures

***Figure 1*: Phylogenic analysis of various VFTMs (a), and comparison of the inactive and active conformations of mGlu1 (b) and NPRC (c) VFTM dimers. a**) Phylogenetic tree was constructed using the sequences of the VFTMs of the mGlu1 receptor, the amide binding protein (AmiC) from the amidase operon, the NR2A subunit of the rat NMDA receptor, Leucine/Isoleucine/Valine binding protein (LIVBP), the natriuretic peptide receptor type A and C (NPRA and NPRC), the Tyrosine kinase receptor type 1 from *Schistosoma mansoni* (RTK1), and the rat $GABA_{B1}$ and $GABA_{B2}$ subunits. Only branches with bootstrap values higher that 600 are shown. Note that the RTK1 VFTM is closer to the $GABA_B$ VFTMs than to any other VFTMs. **b**) Ribbon view of the inactive (left, pdb: 1ewt) and active (right, pdb: 1ewk) forms of the dimer of mGlu1 VFTMs. Note the change in the relative orientation between the two VFTMs, and the closure of one VFTM (the blue-green one in front), that bring together lobes-II. c) Ribbon view of the inactive (left, pdb: 1jdn) and active (right, pdb: 1jdp) forms of the dimer of NPRC VFTMs. Note that ANP binding at the interface between the two VFTMs brings together lobes-II.

***Figure 2*: Three-dimensional models of the open (a) and closed forms (b) of the $GABA_{B1}$ VFTM and possible covalent linkage of both lobes in a closed form by a disulphide bridge.** Lobe-I and lobe-II are shown in red and blue ribbons, respectively. Residues subjected to mutagenesis are represented. A closer view of these residues in the closed form model is shown in **c**. In **d** this same region is shown for the CC1 mutant receptor where Ser247 and Thr315 were mutated into Cysteines.

***Figure 3*: The double cysteine mutants display a DTT-sensitive constitutive activity.** Basal and GABA (1mM)-induced IP formation (black and white columns, respectively) were measured in cells expressed wild-type or double cysteine $GABA_{B1}$ mutants, CC1 or CC2 (**a**) or single cysteine mutants S247C, T316C, E316C (**b**). **c**) Same as in **a**) but GTPγS binding was measured in membranes prepared from cells expressing the indicated $GABA_{B1}$ subunit, the wild-type $GABA_{B2}$ subunit and the Go1 α subunit. **d**) Same as in **a**) but after a 30 min treatment with 10mM DTT (pH 8). Data are expressed as the percentage of the response obtained with the wild-type receptor after 1mM GABA stimulation and are means ± s.e.m. of 3 independent experiments (**a, b** and **d**) performed in triplicate. In **c**) data are means ± s.e.m. of triplicate determinations from one typical experiment.

***Figure 4*: Constitutive activity of CC mutants is not inhibited by the $GABA_B$ competitive antagonist CGP64213. a)** The high basal IP production measured with the CC mutants (black columns) is not inhibited by the $GABA_B$ antagonist CGP64213 (300nM, grey columns), in contrast to the basal activity of the wild-type receptor. **b)** CGP64213 antagonises with the same potency GABA (10µM)-stimulated IP formation in cells expressing the wild-type $GABA_B$ receptor under control condition (black circles) or after DTT treatment (grey circles), or in cells expressing the CC1 mutant after DTT treatment (grey squares). Data are means ± sem of triplicates from one representative out of 3 independent experiments.

***Figure 5*: Cells expressing CC1 remain sensitive to both GABA and CGP64213 with the same potency as cells expressing wild-type receptor.** Concentration-dependent effect of GABA (a) or CGP64213 (in the presence of 10µM GABA) (b) on

IP production in cells expressing wild-type (circles, dashed line) or CC1 (squares, solid line) $GABA_B$ receptor. Data are means ± sem of triplicates from one representative out of 3 independent experiments.

***Figure 6*: Cells expressing CC1 $GABA_B$ receptors possess CGP64213 binding sites at their surface, and new sites are unmasked after DTT treatment**. **a)** [^{125}I]-CGP64213 binding under control condition (white column) or after DTT treatment (white column) on intact cells expressing $GABA_{B2}$ and wild-type or CC1 $GABA_{B1}$ subunits. Data are means of triplicates from one representative out of 3 independent experiments. **b)** Western blot on total membranes of cells mock-transfected, or transfected with plasmid expressing HA-tagged wild-type or CC1 mutant. **c)** Quantification of cell surface expression of wild-type and CC1 mutant $GABA_{B1}$ subunit using an ELISA assay in HEK293 transfected cells.

***Figure 7*: Neither oxidising treatment, nor over-night incubation with agonist or antagonist increase the constitutive activity.** Basal and GABA (1mM)-induced IP formation (black and white columns, respectively) were measured under control (CTR) or after various treatment: DTNB 0.5mM for 30min, CuP (CuSo4 1mM, Phenanthroline 4mM) for 30 min, CuP in presence of GABA 10µM for 30 min, and overnight incubation with CGP64213 (100nM) or GABA (1mM) applied immediately after transfection.

***Figure 8*: Constitutive activity of CC1 mutant receptors is close to GABA-mediated activity of the wild-type receptor.** Basal (diamonds) and GABA-

mediated (squares) IP formation were measured in cells transfected with HA-tagged wild-type (open symbols, thin lines) or CC1 mutant (filled symbols, bold lines) $GABA_{B1}$ subunit (2µg), and various amounts of $GABA_{B2}$ expressing plasmids (0.05 to 2µg). The amount of $GABA_B$ receptors at the cell surface was measured using ELISA and a HA-antibody. Basal IP formation in cells expressing various amounts of CC1 mutant containing $GABA_B$ receptor was also measured after DTT treatment (closed triangles). Data are means ± sem of triplicate determinations from a single experiment. Similar data were obtained in 3 independent experiments. Data points were fitted according to the saturation equation: IP = IPmax . Exp / (Cte + Exp) where IP is the amount of IPs produced under the indicated condition; IPmax is the maximal IP response obtained with high expression level of the receptor; Exp is the signal obtained with the ELISA; Cte is a constant.

***Figure 9*: Migration of the VFTMs of $GABA_{B1}$ and $GABA_{B2}$ is affected by the presence of the two additional cysteines.** Total proteins from cells expressing the wild-type or the CC version of ΔGB1 or ΔGB2 ($GABA_{B1}$ and $GABA_{B2}$ in which a stoip codon has been introduced after TM1) were separated on an acrylamide gel under non-reducing condition, or after treatment with 100 mM DTT pH 8. After transfert, the proteins were detected using an anti HA-antibody directed against the HA epitope inserted after the signal peptide. Data shown are representative of 3 independent experiments.

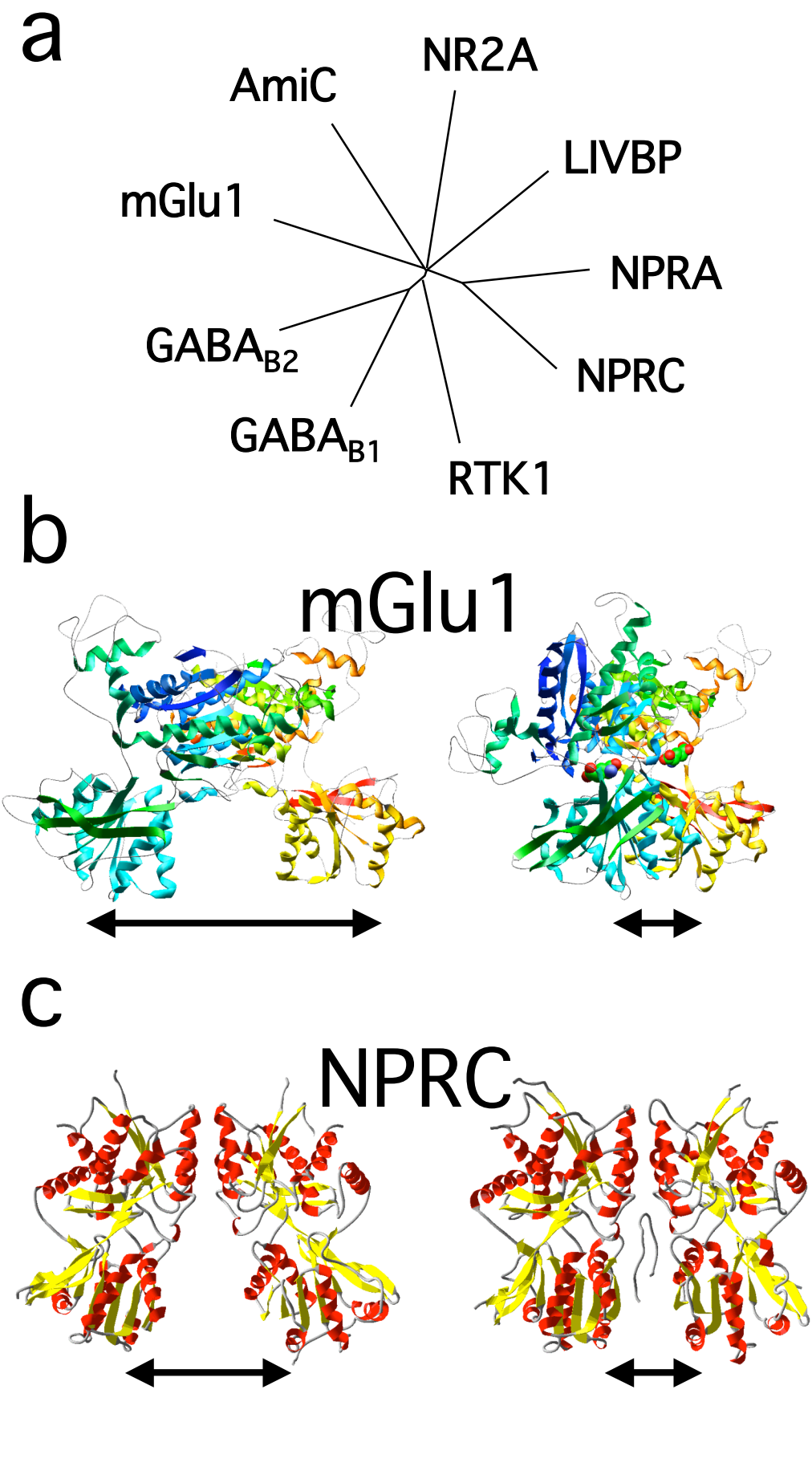
***Figure 10*: Schematic representation of the possible activation process of the heterodimeric $GABA_B$ receptor.** Agonist binding in the $GABA_{B1}$ VFTM (grey) stabilises its closed state, leading to an intermediate unstable state (shown in

parenthesis). This closed state then allows interaction of the VFTMs at the level of their lobe-II, leading to a new relative orientation of the two VFTMs in the dimer. This in turns stabilises a new conformation of the dimer of 7TM domains, leading to G-protein activation. In agreement with this model, locking the $GABA_{B1}$ VFTM in a closed conformation by introducing a disulphide bond is sufficient to lock the entire heteromeric complex into its active state. Whether the $GABA_{B2}$ VFTM (hatched) is in an open or closed state or changes conformation during the activation process is not known.

Table I: Potencies of the agonist GABA, and the antagonist CGP64213 on wild-type (WT), CC1, CC2 and the single cysteine mutants measured either with a functional assay (IP production) or by displacement of [^{125}I]-CGP64213 binding under control condition or after DTT treatment. Values are means ± sem of at least 3 independent experiments. n.t.: not tested.

	IPs production				[^{125}I]-CGP64213 binding			
	CONTROL		DTT		CONTROL		DTT	
	GABA EC_{50} (μM)	CGP64213 IC_{50} (nM)	GABA EC_{50} (μM)	CGP64213 IC_{50} (nM)	GABA Ki (μM)	CGP64213 Ki (nM)	GABA Ki (μM)	CGP64213 Ki (nM)
WT	**0.23 ± 0.02**	**10.5 ± 1.3**	**0.34 ± 0.06**	**12.6 ± 0.8**	**4.74 ± 0.61**	**1.28 ± 0.13**	**2.75 ± 0.97**	**1.53 ± 0.21**
CC1	0.43 ± 0.07	13.1 ± 2.7	0.26 ± 0.08	10.3 ± 0.3	n.t.	3.61 ± 0.81	1.59 ± 0.34	3.43 ± 1.00
CC2	0.33 ± 0.07	12.8 ± 0.7	0.29 ± 0.05	12.0 ± 0.7	n.t.	2.80 ± 0.96	n.t.	n.t.
S247C	0.36 ± 0.06	n.t.	n.t.	n.t.	8.41 ± 0.80	1.67 ± 0.73	n.t.	n.t.
T315C	0.28 ± 0.06	n.t.	n.t.	n.t.	9.50 ± 0.46	1.88 ± 0.98	n.t.	n.t.
E316C	0.85 ±0 .17	n.t.	n.t.	n.t.	5.92 ± 1.5	2.13 ± 0.73	n.t.	n.t.

Figure 1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AmiC
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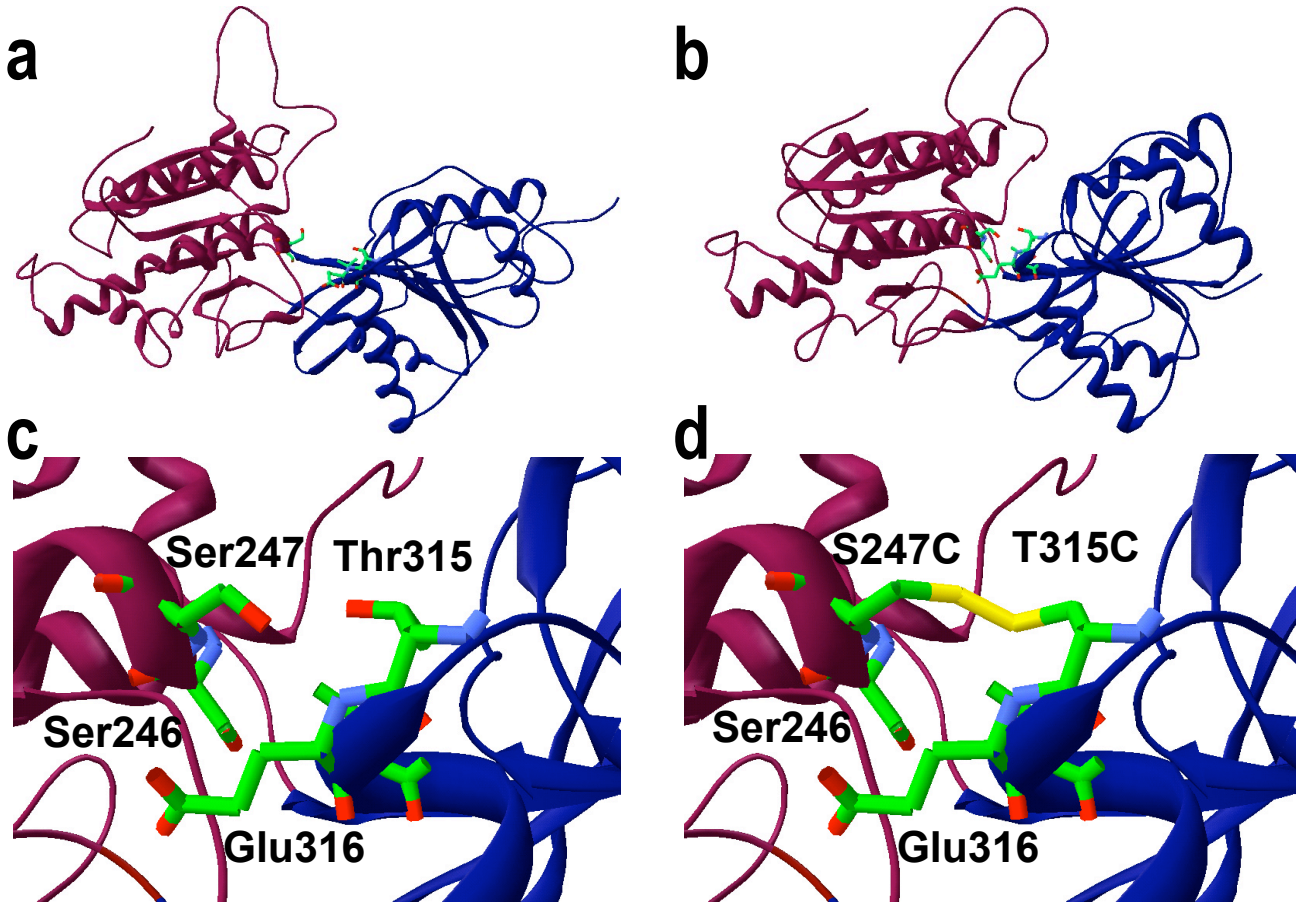
c
NPRC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b
c
d
Ser247
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Glu316
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Ser246
Glu316

Figure 2

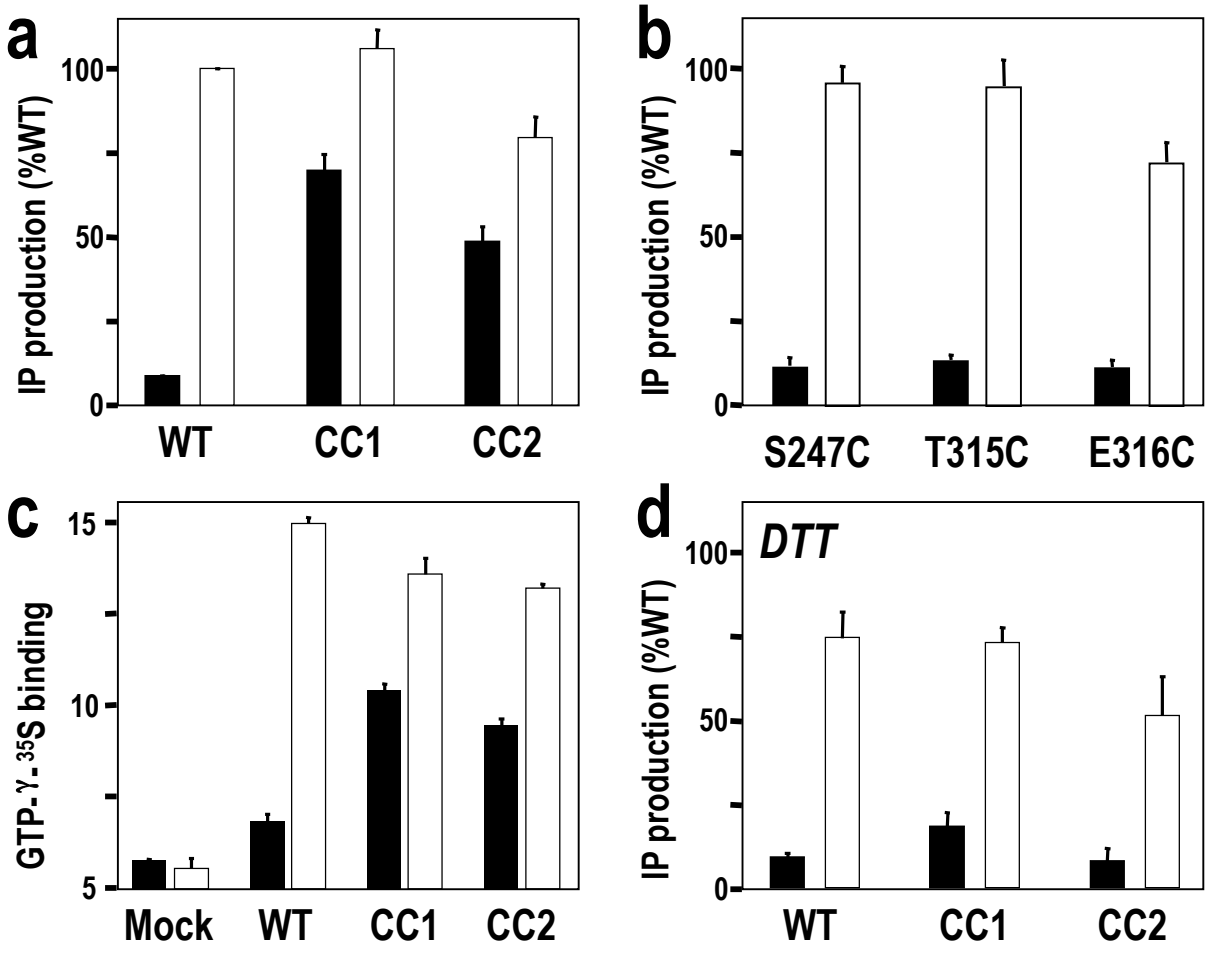
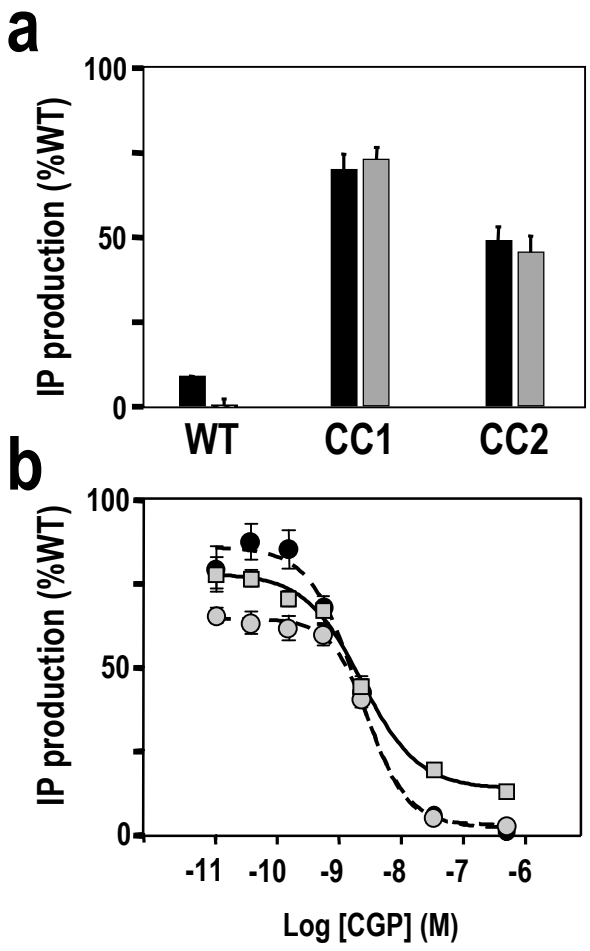

a
IP production (%WT)
100
50
0
WT
CC1
CC2
b
IP production (%WT)
100
50
0
S247C
T315C
E316C
c
GTP-γ-35S binding
15
10
5
Mock
WT
CC1
CC2
d
DTT
IP production (%WT)
100
50
0
WT
CC1
CC2

Figure 3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100
50
0
IP production (%WT)
WT
CC1
CC2
b
100
50
0
IP production (%WT)
-11
-10
-9
-8
-7
-6
Log [CGP] (M)

Figure 4

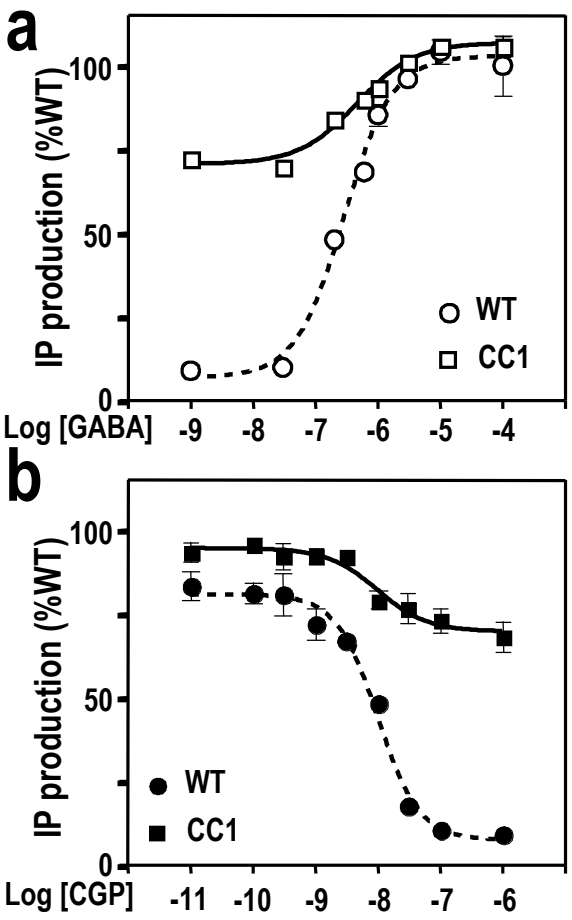
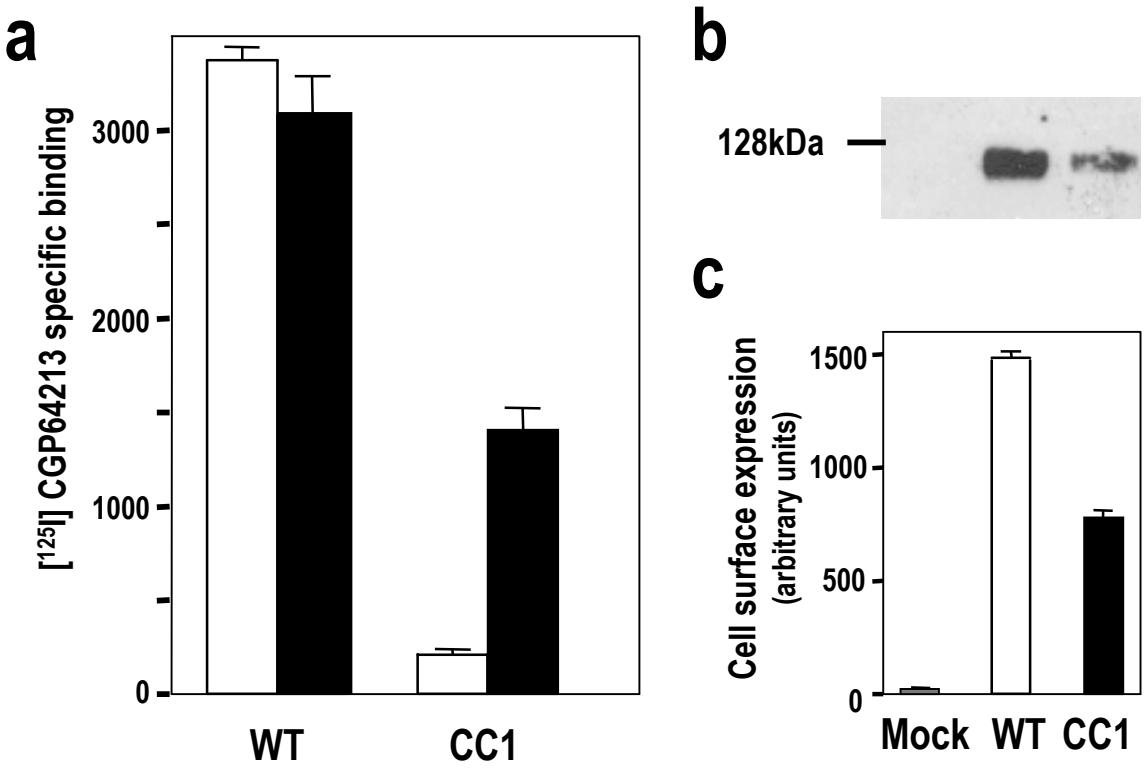

a
IP production (%WT)
100
50
0
WT
CC1
Log [GABA]
-9
-8
-7
-6
-5
-4
b
IP production (%WT)
100
50
0
WT
CC1
Log [CGP]
-11
-10
-9
-8
-7
-6

Figure 5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[125I] CGP64213 specific binding
3000
2000
1000
0
WT
CC1
b
128kDa
c
Cell surface expression
(arbitrary units)
1500
1000
500
0
Mock
WT
CC1

Figure 6

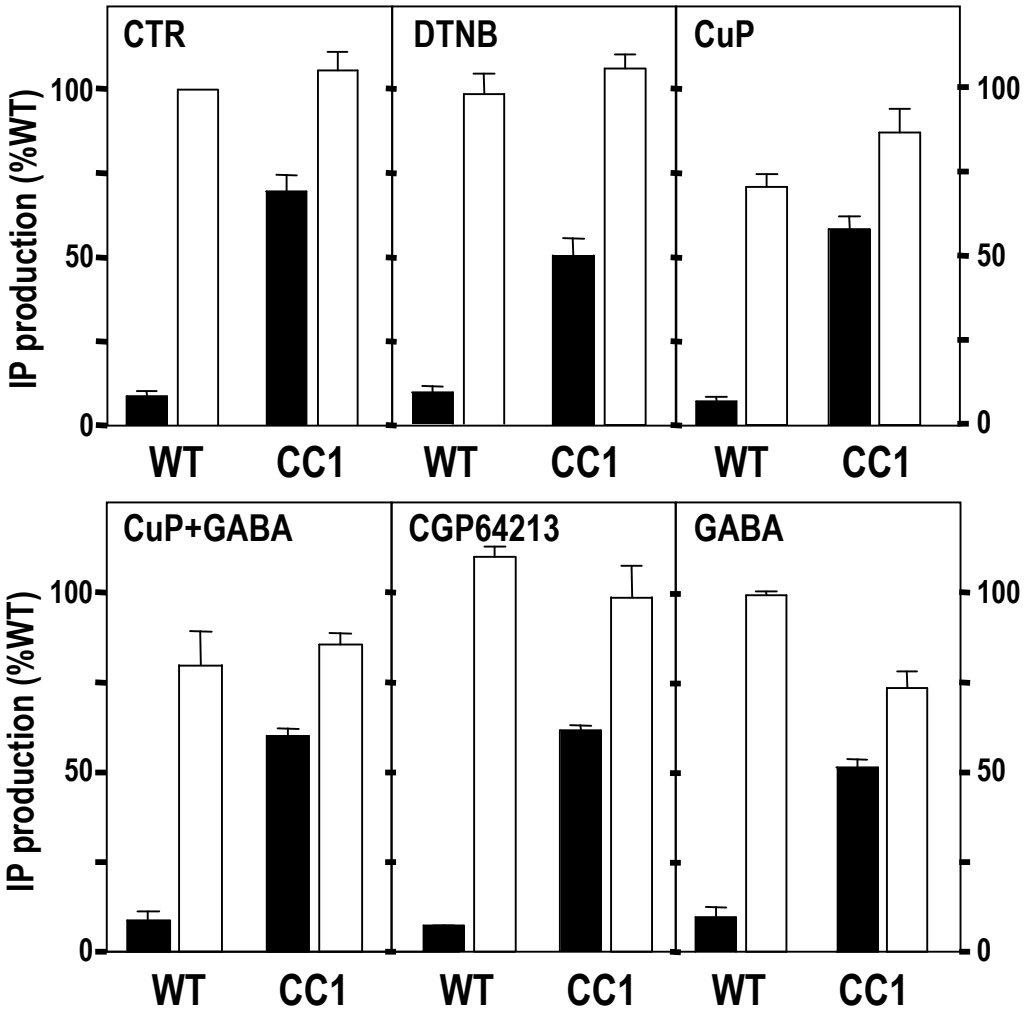
CTR
DTNB
CuP
CuP+GABA
CGP64213
GABA
IP production (%WT)
100
50
0
WT
CC1

Figure 7

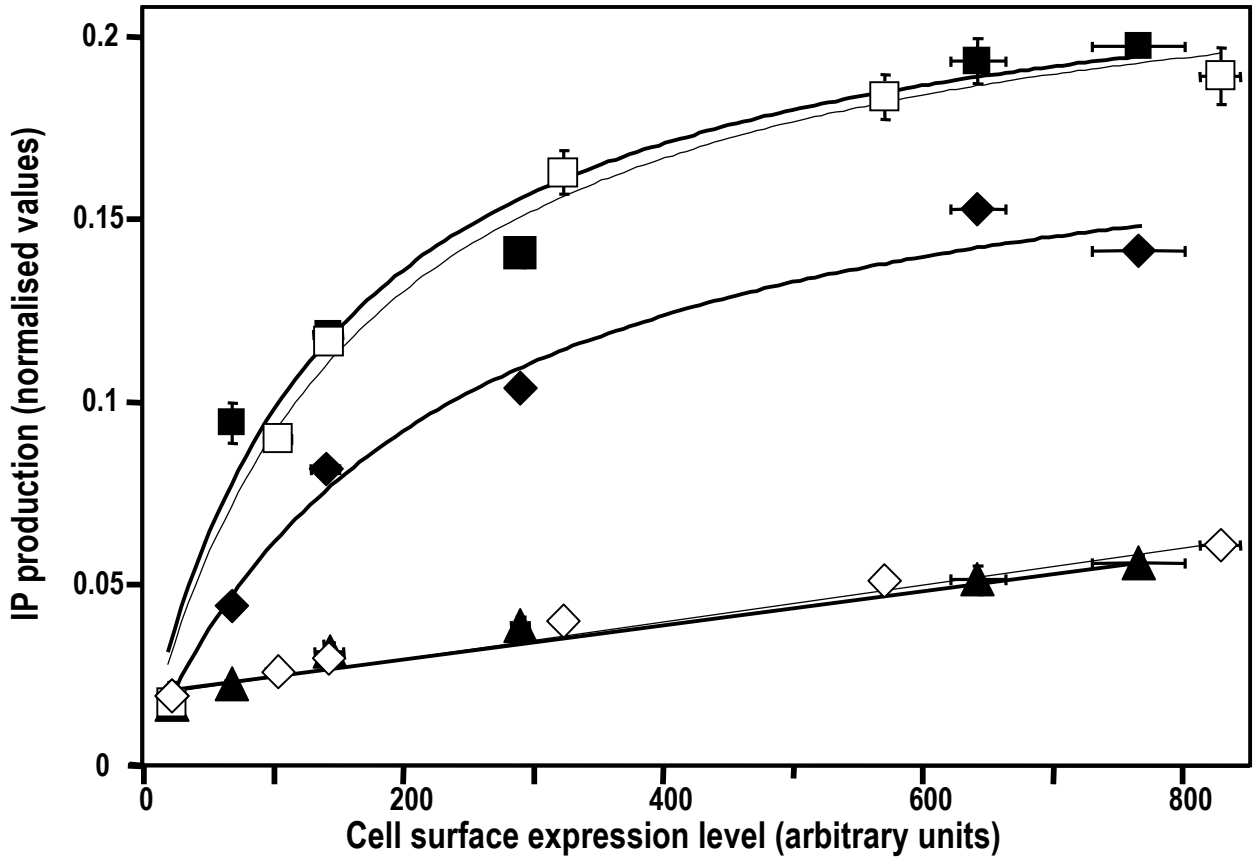
0.2
0.15
0.1
0.05
0
IP production (normalised values)
0
200
400
600
800
Cell surface expression level (arbitrary units)

Figure 8

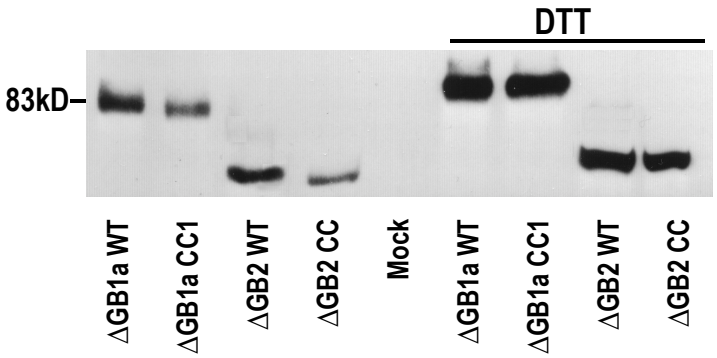
DTT
83kD
ΔGB1a WT
ΔGB1a CC1
ΔGB2 WT
ΔGB2 CC
Mock
ΔGB1a WT
ΔGB1a CC1
ΔGB2 WT
ΔGB2 CC

Figure 9

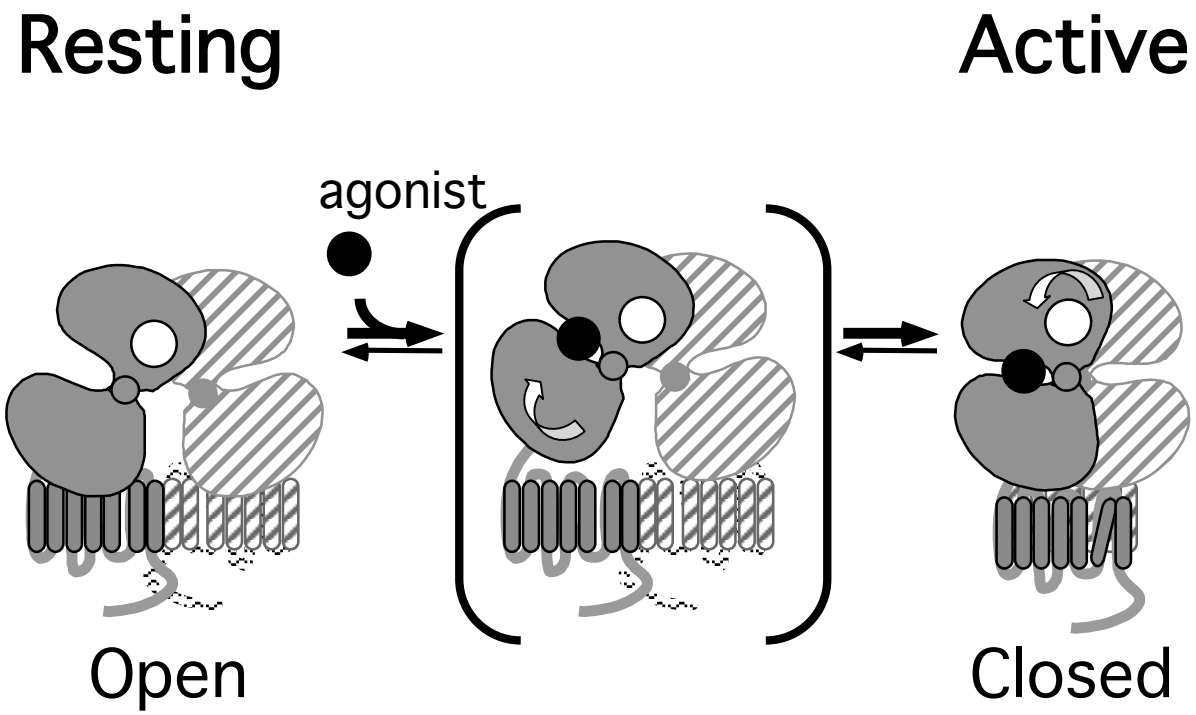

Figure 10