G Protein-Coupled Receptors: Dominant Players in Cell-Cell Communication

Joël Bockaert, Sylvie Claeysen, Carine Becamel, Sylvie Pinloche, Aline Dumuis

G-Protein-Coupled Receptors (GPCRs): dominant players in cell-cell communication

Joël Bockaert, Sylvie Claeysen, Carine Bécamel, Sylvie Pinloche* and Aline Dumuis

UPR CNRS 9023 – 141 Rue de la Cardonille – 34094 Montpellier Cedex 5, France

Tel: +33 467 142930 – Fax: +33 467 542432 – e.mail: bockaert@ccipe.montp.inserm.fr

*LIRMM, 161 rue Ada, 34392 Montpellier Cedex 5, FRANCE

Abstract

The G-protein-coupled receptors (GPCRs) are the most numerous and the most diverse type of receptors (1 to 5 % of the complete invertebrate and vertebrate genomes). They transduce messages as different as odorants, nucleotides, nucleosides, peptides, lipids and proteins. There are at least eight families of GPCRs showing no sequence similarities and using amazing different domains to bind ligands and activate a similar set of G-proteins. Homo- and hetero-dimerization of GPCRs seem to be the rule and in some cases, an absolute requirement for activation. There are about 100 orphan GPCRs in the human genome which will be used to find new message molecules. Mutations of GPCRs are responsible for a wide range of genetic diseases. The importance of GPCRs in physiological processes is illustrated by the fact that they are the target of the majority of therapeutical drugs and drugs of abuse.

Key words

G-protein-coupled receptors, G-proteins, orphan receptors, dimerization, constitutive activation, inverse agonism, genetic diseases, cellular signaling, desensitization.

I. Introduction

In 1957, when most of the readers of this review were still at elementary school or perhaps not even born, Sutherland and Rall were busy describing the basic properties of an enzyme, the adenylate cyclase (now called adenylyl cyclase (AC)), its activation by hormones such as epinephrine and glucagon, and its well known product cAMP (Sutherland and Rall, 1958). Ten years later, AC was still believed to be a unique allosteric enzyme regulated by the binding of hormones on a regulatory site. In the late 60s, Rodbell and Birnbaumer demonstrated that fat cell AC is activated by multiple hormone receptors, in a non-additive manner, and they proposed that AC and hormonal receptors are distinct (Birnbaumer and Rodbell, 1969). A proposal which was subsequently demonstrated by Orly and Schramm (Orly and Schramm, 1976). Rodbell and Birnbaumer also demonstrated that receptors need a third partner to activate AC, the GTP-binding protein (G-protein). G-proteins plays the go-between receptors and AC (Rodbell *et al.,* 1971). In 1983, Gs (the G-protein which activates the AC) was the first G-protein to be purified by Gilman and co-workers (Northup *et al.*, 1983). Purification of rhodopsin, the GPCR activated by light was achieved in 1967 (Shields *et al.*, 1967) whereas the first purification of a hormonal GPCR (the β-adrenergic receptor, β-AR) was carried out by Lefkowitz's team in 1981 (Shorr *et al.*, 1981).

Twenty years later, GPCRs constitute one of the largest known categories of proteins. Based on the fact that these receptors are all formed of seven transmembrane domains (TM-I to TM-VII) and are believed to activate G-proteins, GPCRs are generally assumed to form a single super-family (Baldwin, 1993; Bockaert and Pin, 1998; Bockaert and Pin, 1999; Bourne, 1997b; Josefsson, 1999; Kolakowski, 1994; Wess, 1997). However, as we all know, the more data we accumulate, the more complex is the picture. Indeed, in this review, we will see that : (1) the comparison of primary amino-acid sequences leads to the multiplication of GPCR

families (up to 8 families can be recognized to date) (Bockaert and Pin, 1999; Josefsson, 1999); (2) some GPCRs may signal by interacting with non G-proteins, in addition or not to their G-protein signaling (Hall *et al.,* 1999). Calling some of them GPCRs may therefore be inaccurate. A large number of seven transmembrane proteins, (such as latrophilin, the receptor for the spider toxin α−latrotoxin), EMR1 (human cell surface glycoprotein F4/80), BAI-1 (brain-specific angio-genesis inhibitor), or Methuselah, classified as LNB-TM7 (seven TM domains containing a long N-terminal extracellular region) have been described (Stacey *et al.,* 2000). The long N-terminal domain of LNB-TM7 contains several repeats such as EGF repeats, thrombospondin type 1 repeats, olfactomedin homologous regions, galactose-binding lectin homologous region and laminin AG-type repeats. If the coupling of LNB-TM7 proteins to G-proteins exists, it has only been demonstrated for latrophilin (Stacey *et al.,* 2000). More recently, another seven transmembrane protein having 20-25% identity with the core domain of family 3 GPCRs, but devoid of the long and specific extracellular domain which is the characteristic of this family (see figure 4), has been cloned (Brauner-Osborne and Krogsgaard-Larsen, 2000). Whether or not it is a GPCR, remains to be demonstrated.

GPCRs clearly represent some of the oldest devices devoted to signal transduction, present in protozoa and earliest diploblastic metazoa (New and Wong, 1998; Vernier *et al.,* 1995), slime mold (Devreotes, 1994), yeast (Dohlman *et al.,* 1991), plants (Josefsson and Rask, 1997; Plakidou-Dymock *et al.,* 1998), invertebrates (Bargmann, 1998; Rubin *et al.,* 2000) and vertebrates (Bockaert and Pin, 1999). GPCRs represent 5% *Caenorhabditis elegans* (1100 genes) (Bargmann, 1998), 1% Drosophila (160 genes) (Rubin *et al.,* 2000) and certainly 1-3% of vertebrate genomes (more than 1000 genes) (Bockaert and Pin, 1999).

During evolution, "molecular tinkering" of GPCR genes has been used to adapt their structure to recognize a wide range of environmental stimuli and regulatory molecules involved in cell-cell communication. The success of this adaptation was tremendous (Bockaert and Pin, 1999;

Yokoyama *et al.*, 1999). GPCRs are implicated in recognizing messages as diverse as light (Shichida and Imai, 1998), Ca^{2+}, odorants of different chemical nature, amino acids, peptides, lipids, nucleotides, as well as proteins (figure 1A). They control the activity of effectors as diverse as enzymes (AC, phosphodiesterases, phospholipases…), ionic channels (Ca^{2+}, K^{+}, Cl^{-} …), transport vesicles, kinases and are key elements in physiological functions such as morphological movements during gastrulation and germ band extension, hormonal regulatory systems, synaptic transmission, and recognition of sensory stimuli (light, odorants, taste molecules, as well as pain stimuli). The odorant GPCRs are certainly the most abundant ones in most organisms representing roughly 50% of the total number of GPCRs in Drosophila and vertebrates and as much as 90% in *Caenorhabditis elegans*.

Such an important device for cell-cell communication in multi-cellular organisms, has been a key element during their evolution. In addition, GPCRs are direct or indirect targets of the vast majority of available therapeutics drugs, as well as drugs of abuse (Roush, 1996; Stadel *et al.,* 1997). Somatic and genetic mutations of GPCRs generate various pathologies (Birnbaumer, 1995; Spiegel, 1996; Van Sande *et al.,* 1995).

Since several recent reviews are available (Bockaert and Pin, 1999; Gether, 2000; Wess, 1997), we will summarize here most of the biochemical, pharmacological and physiological knowledge of this old (almost 50 years…), but still rapidly growing field.

II. GPCR signal transduction: from a "ménage à trois" to a "ménage à quatre"

A. Rodbell's model: the receptor, the transducer (G-protein) and the effector

We have already recalled the crucial experiments Rodbell performed on fat cells during the late 60's (Birnbaumer and Rodbell, 1969). The non-additivity of several hormonal receptors

on AC activity posed a problem for M. Rodbell who thought it inconceivable that several hormonal receptors could structurally anneal to the same enzyme (Rodbell, 1995). After long discussions with O. Hechter, M. Rodbell introduced the concept of hormone action as "a ménage à trois". This concept which is still valid today, was based on cybernetic theories of information introduced by N. Wiener (Weiner, 1961). The receptor was the equivalent of the discriminator, the AC was the amplifier and the notion of transducer was introduced. The transducer was a coupling device designed to allow communication between the discriminator and the amplifier. At that time, the nature of the transducer was unknown and Rodbell had no idea that it could be a GTP binding protein. This idea came a few months later after a series of experiments when the team observed that the binding of the hormone (the ^{125}I-glucagon) was acutely modified (increase in k_{on} and k_{off}, reduction of the "steady state" binding level) by some components used in the AC assay. After a clever search for the "culprit", they found that it was GTP. Not only GTP is modifying the hormonal binding characteristics, but they found that GTP was an absolute requirement to observe, *in vitro*, the stimulation of AC by receptors: the transducer was a GTP binding protein (G-protein) (Rodbell, 1992).

The purification of the G-protein by A. Gilman (Northup *et al.*, 1983) revealed the trimeric nature of G-proteins. In their inactive form (state 1, figure 1B), the three subunits (Gα, Gβ, Gγ) are associated and the Gα subunit binds GDP. The Gβγ complex is never dissociated under biological conditions and can be considered as a single unity. GPCRs can be considered as releasing exchange factors, functions homologous to those of monomeric small G-proteins GRF/GEF (GRF: GDP releasing factors or GEF: guanine nucleotide exchange factor). Their role is to catalyze the GDP release from Gα. State 2 is then obtained (figure 1B) and is characterized as an empty nucleotide site. The Gα and GPCR have different structures than in states 1 and 3; they are tightly associated (Chabre and Deterre, 1989). The GPCR affinity for agonists is generally higher than under states 1 and 3. It corresponds to the high agonist

affinity state obtained in binding experiments when performed *in vitro* in the absence of GTP. State 2 is a short-life transition state. *In vivo*, GTP rapidly associates with the Gα empty state; dissociation then occurs. The Gα-GTP and Gβγ are split and dissociated from the activating GPCR (state 3, figure 1B). Gα and Gβγ can activate their respective effectors (figure 1B) (Bockaert and Pin, 1998; Clapham and Neer, 1993; Hamm, 1998). In some cases, both Gα and Gβγ are needed to activate their effector. This is the case for the activation of type II, IV and presumably type VII AC (Defer *et al.*, 2000; Tang and A.G., 1991) and some L-type Ca^{2+} channels (figure 1B). GTP hydrolysis is the only event that stops the activation of both Gα-GTP and Gβγ. The re-association of Gβγ with Gα-GDP also stops the action of Gβγ on their effectors. GTP hydrolysis is certainly tightly controlled, especially by proteins like RGS (regulators of G-protein signaling) as discussed in section II-B (de Vries *et al.*, 2000).

There are 17 genes (leading to at least 23 proteins) coding for Gα, 5 for Gβ and 12 for Gγ (Hamm, 1998). Not all the conceivable heterotrimeric Gαβγ complexes are indeed formed in reality. This can be due to steric problems especially for the GβGγ association (Lee *et al.*, 1995). The Gα proteins are divided into 4 families based on sequence similarities (figure 1B):

- the Gαs family which also comprises Gαolf (localized in olfactory neurons but also in striatum);
- the Gαi family which comprises Gαt, the transducin implicated in visual transduction, Gα-gus, expressed in gustative neurons but also Gαo and Gαz;
- the Gαq family which also contains Gα11, Gα14, Gα15 and Gα16. Gα15 and Gα16 are homologous proteins expressed in hematopoïetic cells of rodents and human respectively (Gomeza *et al.*, 1996b; Offermanns and Simon, 1995; Parmentier *et al.*, 1998). They have the remarkable property of being stimulated by most GPCRs, those which are naturally coupled to Gq but also those coupled to Gi/Go and even Gs;
- the Gα12,Gα13. family.

The crystal structures of Gα (Gαt and Gαi), as well as Gαβγ, have been obtained at a resolution of 2-3Å, in (a) an inactive form (GDP form) (Lambright *et al.,* 1994; Lambright *et al.,* 1996; Mixon *et al.,* 1995; Wall *et al.,* 1995) (state 1, figure 1B); (b) under a transition mimicking state (GDP-AlF4-) associated (Tesmer *et al.,* 1997a), or not (Sondek *et al.,* 1994) with RGS4; and (c) in an active state (Gαs-GTPγS), associated (Tesmer *et al.,* 1997b), or not (Coleman *et al.,* 1994; Noel *et al.,* 1993) with the catalytic domains of AC. Gα subunits contain two domains, a domain involved in binding and GTP hydrolysis (the G domain) which is structurally similar to the super-family of GTPases, including small G-proteins and elongation factors (called the "ras"-like domain), and a unique helical domain (hα) that is inserted within the ras domain, which buries GTP in a pocket (figure 2A, B). The Gβ subunits have a 7-membered β-propeller structure based on 7 WD-40 repeats. Gγ subunits interact with Gβ through their N-terminal coiled-coil and all along Gβ. Gα subunits are myristoylated and/or palmitoylated at their N-terminus, and Gγ is farnesylated or geranyl-geranylated at its C-terminal. These acyl and prenyl groups are the only devices controlling the association between the heterotrimeric Gαβγ complex with the membrane. In section V, we will discuss the mechanism by which GPCRs interact and activate G-proteins.

Reversible palmitoylation may possibly play a regulatory role in GPCR action (Wedegaertner and Bourne, 1994). Long-term modulation of G-protein mRNA levels has been reported (Milligan, 1993), but at protein levels, G-proteins are generally stable molecules with long lives (Brabet *et al.,* 1991).

B. New partners in GPCR activation of G-proteins: the RGS

For many years now, it has been known that Ras-type, monomeric, small G-proteins have a very low GTPase activity. Specific proteins called GAP (GTPase activating proteins) are an absolute requirement for the deactivation of these proteins (Boguski and McCormick, 1993).

Any reduction in the capacity of GAP to activate Ras GTPase activity is a common cause of malignancies. The mechanism of activation of Ras GTPase activity by GAP involves the coordination between a Gln (Q^{61} often mutated in oncogenic Ras) and a Thr (T^{35}) of Ras, a glutamic acid residue of GAP protein and a water molecule. The GAP protein puts its glutamic "finger" inside the Ras protein (Bourne, 1997a). Interestingly, in heterotrimeric G-proteins, which have an intrinsic GTPase activity 1000-fold higher than the small G-proteins, corresponding Gln (Q^{204} in Gαi, Q^{227} in Gαs) and Thr (T^{182} in Gαi and T^{204} in Gαs) coordinate a glutamic acid "finger" localized within the molecule itself (R^{178} in Gαi and R^{201} in Gαs localized in linker 2) and a water molecule to hydrolyze the GTP molecule. Note that mutations of the corresponding Gln and Arg in Gi or Gs have been found in tumors, and pathologies such as McCune-Albright and pseudo-hypoparathyroidism. In addition, ADP-ribosylation of the R^{201} of Gs by cholera-toxin reduces its GTPase activity leaving the protein to be constitutively active. Overproduction of cAMP within the intestinal epithelial cells is responsible for diarrheas (Spiegel, 1996; Spiegel *et al.,* 1993).

For a long time, GTPase activity of purified heterotrimeric G-proteins was recognized to be still too slow to explain the deactivation of cellular processes under the regulation of G-proteins. The t1/2 of hydrolysis of GTP by most Gα proteins is in the 10-20s range (Gilman, 1987) and even higher for Gαq (50s) and Gαz (7min) (see Berstein *et al.,* 1992). The GTP hydrolysis on purified transducin (Gαt) is close to 15s, although the deactivation of visual signals is close to 0.2s (Navon and Fung, 1984; Vuong and Chabre, 1990). The Gαt effector, GMPc phosphodiesterase, has been proposed to be the GAP which accelerates GTP hydrolysis, but this is still a matter of controversy (Antonny *et al.,* 1993; Arshavsky and Bownds, 1992). The idea that the effector can serve as GAP came from the fact that the effector of Gαq, the phospholipase C β1, is a GAP (Berstein *et al.*, 1992). The effector, and in particular the phosphodiesterase (PDE) of rods, clearly act in synergy with the real GAP of

Gαt, the RGS9 both *in vitro* and *in vivo* (He *et al.*, 1998; Tsang *et al.*, 1998). The discovery of RGS came simultaneously from genetic studies and double hybrid studies using Gαi3 as bait. In the latter, de Vries et al. were able to "fish" for a protein that they called GAIP (Gα interacting protein) (De Vries *et al.*, 1995; de Vries *et al.*, 2000; Dohlman and Thorner, 1997). In *Caenorhabditis elegans*, and *Sacchromyces cerevisiae*, mutations within two genes, EGL-10 and SST2, respectively, were responsible for a delay in egg laying and reduced desensitization to pheromones respectively (de Vries *et al.*, 2000; Dohlman and Thorner, 1997). In fact, the three proteins, GAIP, EGL-10 and SST2 were homologous within a 130 residue domain, now called the RGS domain (de Vries *et al.*, 2000; Wieland and Chen, 1999). This domain is responsible for the binding to Gα proteins. RGS accelerate 50 to 100 times GTP hydrolysis of Gα proteins which reduces the t1/2 of GTP hydrolysis to a value compatible with the "physiology" (Berman *et al.*, 1996).

There are now 30 mammalian RGS proteins (de Vries *et al.*, 2000), which differ considerably in size (17 to 160 kDa). The mechanism of action of RGS is certainly still in its infancy. The known role of RGS is of course to have a GAP function. This is confirmed *in vitro* but also *in vivo* although mainly in recombinant systems. RGS clearly attenuate the action of GPCRs as shown with RGS4 and GAIP which inhibit the transduction of bradykinin (Gαq-mediated) and somatostatin (Gαi-mediated) receptors (de Vries *et al.*, 2000). In addition, the binding of RGS to Gα, results in an inhibition of the effectors–Gα associations. RGS4 is able to inhibit GTPγS-activated PLC-β1. Similarly, PLC-β1 can displace RGS4 bound to Gαq-GDP-AlF4 (de Vries *et al.*, 2000). Finally, as already discussed, the increase in Gα GTP hydrolysis and therefore accumulation of Gα-GDP levels, accelerates the deactivation of GPCR transducing effects mediated via βγ. Over-expression of RGS1, RGS2, RGS4, and RGS8, accelerates the turning off of G-protein-coupled inward rectifying K^+ channels (GIRKs), known to be activated by βγ (Doupnik *et al.*, 1997). More surprisingly, RGS also result in an increase in

the k_{on} of GIRK activation by βγ (Doupnik *et al.*, 1997). Rod photo-response recovery is slowed in mice lacking RGS9-1 (Chen *et al.*, 2000). RGS is made of 9 α−helical structures that form two lobes, one made of helical structures α1, α2, α3, α8, α9, and the other made of helical structures α4, α5, α6, α7 (Tesmer *et al.*, 1997a). The base of α3−α4, α5−α6 and the α7−α8 loops, constitutes the contacting surface with the Gα subunit. The latter is made by the N-terminal domain of Gα, switches I and II, and to a lesser extent switch III (switch regions are the domains where there is a dramatic conformational change during the GTP cycle (see figure 2) (Sprang, 1997). Switch II is also part of the interacting surface of Gα with βγ and the effector AC as revealed in the co-crystal Gαs-GTPγS-AC (Tesmer *et al.*, 1997b). However, crystallographic data indicate that Gαi can accommodate RGS4 and AC at the same time (Sunahara *et al.*, 1997). Similarly, the synergism between the effect of RGS9 and PDEγ on the GTPase activity of Gαt, suggests that both the effector and RGS can interact simultaneously with some Gα (He *et al.*, 1998). This is *a priori* somehow contradictory with the idea that RGS can antagonize the Gα−effector association. The stabilization of the switch regions, and in particular switch II, in which the important Arg residue implicated in GTP hydrolysis is localized (see early discussion), is certainly the basis of RGS action (Natochin *et al.*, 1998). Several residues of RGS4 (S^{85}, E^{87}, N^{88}, L^{159}, D^{163}, S^{164}, R^{167}) encircle the Thr (T^{182} in Gαi1) engaged in GTP hydrolysis. N^{88}, L^{159} and R^{167} seem to be the most important for the formation of the Thr^{182} pocket (Druey and Kehrl, 1997). However, more work has to be done to understand the structural basis of RGS mechanisms of action.

One major question is whether there is specificity in the RGS-Gα interaction. To make things simple, most of the RGS proteins tested so far are GAP for Gi/Go and Gq. p115Rho GEF (and perhaps PDZ-rhoGEF) (PDZ: a 90-residue domain first recognized in the post-synaptic density protein **P**SD95, in its Drosophila analog **D**isc-large tumor suppressor gene and in the tight junction protein **Z**O-1) (Kornau *et al.*, 1997; Songyang *et al.*, 1997; Sudol, 1998b) are

specific GAP for G12/G13. No RGS have been found for Gαs (de Vries *et al.*, 2000). This may be due to the fact that the Asp (D^{229}) of Gαs (not conserved in the Gαi family) is localized in switch II, and may constitute a barrier for its interaction with RGS. However, some sub-domains of AC-V have been found to be able to accelerate both activation and deactivation of Gs (Siderovski *et al.,* 1999).

Most RGS are present in the cytosolic pool, in plasma membrane (PM) and in intracellular membrane pools (especially membrane vesicles for GAIP). They can be recruited by Gα at the PM but can also have an intrinsic TM domain (RET-RGS1) and be palmitoylated (GAIP, RGS4). RGS 12 has a PDZ domain which binds the C-terminus of IL-8B and CXCR2 receptors (Snow *et al.*, 1998). RGS 12 also binds and inhibits N-type Ca^{2+} channels (Schiff *et al.*, 2000).

In addition to the RGS domain, RGS contain other structural domains, at the N-terminal and the C-terminal positions. These structural domains connect RGS to other interacting proteins (de Vries *et al.*, 2000).

In conclusion, the "ménage à quatre" in the GPCR transduction is already well established. The implication of RGS in cancer, seizures, and drug tolerance is just starting to be investigated but is certainly promising (de Vries *et al.*, 2000). The regulation of RGS7 by TNF and the endotoxin LPS is also particularly interesting. Indeed, these pro-inflammatory and sleep-inducing compounds inhibit the degradation of RGS7 by the proteasome system in the brain. This effect may certainly lead to a decrease in the Gαi/o-mediated anti-inflammatory GPCR action and an increase in the secondary anti-inflammatory effects of TNF mediated via Gs-associated GPCRs (Benzing *et al.*, 1999).

Another intriguing finding is that newly discovered proteins, such as AGS (activator of G-protein signaling) stimulate the GDP/GTP exchange on Gα proteins (Cismowski *et al.,* 2000). The AGS1 is a ras-related protein possibly making a new link between small G and

heterotrimeric G. Rapsynoid, a Drosophila protein, shares 43% identity with AGS (Parmentier *et al.,* 2000b). RGS14 has been proposed to be a Rap effector (Bidot *et al.,* 2000). As such, the "ménage à quatre" may already be old-fashioned.

III. The different families of GPCRs and their binding sites

Sequence comparison between the different GPCRs has revealed the existence of different receptor families sharing no sequence similarities (Bockaert and Pin, 1998; Bockaert and Pin, 1999; Josefsson, 1999; Kolakowski, 1994). However, all these receptors seem to share a central core domain made up of seven transmembrane domains (TM-I to -VII) which are helical tubes connected by three extracellular loops (E-I to E-III) and 3 cytoplasmic loops (C-I to C-III). Within the C-terminus, a C-IV loop has been proposed to be formed between the TM-VII and the cysteine residues that are palmitoylated (figures 1 and 2). The crystal structure of rhodopsin revealed that this loop is an α-helical structure (helice VIII, figure 2D). We have constructed a phylogenic tree of 56 GPCRs taken from diverse eucaryotes including yeast, slime mold (*Dictyostelium discoideum*), and the only GPCR cloned so far in plant is from *Arabidopsis thaliana*. Protein sequences (excluding N-terminal and C-terminal domains) were multi-aligned and a tree was calculated using Clustal W. No further functional driven hypotheses were taken into consideration to adjust the alignment. Using this simple method we were able to classify these receptors into 8 distinct families (figure 3). Only a few receptors were not included in these families, the pheromone yeast GPCRs (STE2 and STE3 and pheromone P), the *Arabidopsis thaliana* putative GPCR (Josefsson and Rask, 1997; Plakidou-Dymock *et al.,* 1998), the Methuselah protein (an LNB-7TM protein of Drosophila) (Lin *et al.,* 1998), the mutation of which extended life-span and stress resistance, and the Drosophila putative taste receptors (GR = gustatory receptor (Clyne *et al.,* 2000) family). In

addition, these receptors did not group together. In this section, we will not describe the receptor for trehalose, recently cloned in Drosophila which may represent a new subclass or family, (Clyne *et al.*, 2000).

We (Bockaert and Pin, 1998; Bockaert and Pin, 1999) and others have previously described 6 families of GPCRs, using generally more polished alignments (for example; without the intra- and extracellular loops). These families are again found in the present study: Family 1: the rhodopsin receptor family, family 2: the PTH, PACAP, VIP receptor family, family 3: the glutamate metabotropic receptor (mGluR) family, family 4: one vomeronasal pheromone receptor family (VN), family 5: the frizzled receptor family, and family 6, the cAMP receptor family. There are now two other families, family 7: the vertebrate T2R gustative receptor family which responds to bitter tastants (Adler *et al.,* 2000; Chandrashekar *et al.,* 2000) and family 8 which corresponds to a new family of putative odorant receptors in Drosophila (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999).

Receptors from different families share no sequence similarity, suggesting that we are in the presence of a remarkable example of molecular convergence.

Family 1: contains most of the GPCRs including receptors for odorants (more than one thousand). One important recent event concerning this family was published in 2000. This is the crystal structure (2.8Å) of rhodopsin (figure 2D) (Palczewski *et al.,* 2000). As expected from previous electron diffraction structures of rhodopsin (Unger *et al.,* 1997), the TM-I, IV, VI and VII are bent at Pro (P) residues, although in TM-I it is not significant and in TM-IV it is present at the extracellular end. Prolines are conserved in all members and play a crucial role in activation. TM-V with a proline in its middle is almost straight, while TM-II has a kink in the middle at the Gly-Gly doublet. TM-IV and VII are shorter than the other TMs.

The following residues and sequences are considered to represent the signature of this family:

(a) an Asp (D) of TM-II which is essential for the coupling to G-proteins and which can form a hydrogen bond with the side chain of a conserved Asn (N) in TM-I and an Ala (A) in TM-VII in rhodopsin (Palczewski *et al.*, 2000). In other receptors, the Asp in TM-II may have a possible contact with an Asn in TM-VII. Complementing mutations in many receptors argue for such a direct interaction. In any case, TM-I, TM-II and TM-VII are in close contact (figure 2D) (Zhou *et al.*, 1994; Zhou and Fishman, 1991);

(b) the Asp-Arg-Tyr (DRY) or Glu-Arg-Tyr (ERY) or Glu-Arg-Tryp (ERW) sequences found at the N-terminus of C-I are highly conserved and are directly involved in G-protein activation (Oliviera *et al.*, 1994; Scheer *et al.*, 1996). Mutation of Arg in this sequence suppresses G-protein activation. This sequence is surrounded by hydrophobic residues from H-II (Pro^{71} and Leu^{72} in rhodopsin), C-II (V136V137V138 and Phe^{148} in rhodopsin), H-V (Leu^{226} and Val^{230} in rhodopsin) and H-VI (Val^{250}, Met^{253} in rhodopsin). This region is likely to be a surface contact with G-proteins (Palczewski *et al.*, 2000);

(c) basic residues at the end of C-III (Lys^{245}, Lys^{248}, Arg^{252} in rhodopsin) are currently found at that position crucial for specific activation of G-proteins (Bockaert and Pin, 1999; Bourne, 1997b);

(d) other intra-domain constraints are likely to be important to keep the receptor under an inactive "R" state. This includes a bridge constraint made by Lys^{296} of TM-VII which is covalently attached (Schiff base) to the retinal. The retinal protonated Schiff base also makes a salt bridge with Glu^{113} in TM-III (figure 5). Mutation of Lys^{296} generates a constitutively active receptor and causes *retinitis pigmentosa* (Robinson *et al.*, 1992). In α_2-adrenergic receptors, this salt bridge is likely to be between an Asp of TM-III and a Lys in TM-VII. Mutations of these residues resulted in constitutive activation of the receptors and complementing mutations reverse this phenotype (Porter and Perez, 1999). Interestingly, the binding of the protonated amine of adrenaline probably disturbs the salt bridge due to its

binding to the Asp of TM-III. However, a Lys in TM-VII is not conserved in all biogenic amine receptors. Other constraints include hydrophobic interactions between a conserved Ile-Leu doublet of TM-III and a Phe of TM-VI (mutations of these residues constitutively activate the C5a-receptors) (Baranski *et al.,* 1999), and a bridge between a highly conserved Tyr in TM-VII and Asn in TM-II (Palczewski *et al.*, 2000).

A cysteine-cysteine bridge is very well conserved between E-I and E-2 in all GPCRs of this family. In rhodopsin, the N-terminal domain contains two anti-parallel β-sheets which are located just below the E-II loop, which also contains two anti-parallel β-sheets. All four β-sheets, as well as most parts of E-II, make an extracellular plug blocking the exit from the pocket (Palczewski *et al.,* 2000). The E-II loop is almost inserted within the TM core, whereas the Asn^{2} is above the E-III loop in contact with Asp^{282}. The role of the plug in rhodopsin is not clear, however, it may prevent the all-trans retinal from projecting out of the pocket during activation. It remains to be seen if such plugs are found in other GPCRs of this family.

Depending on the size and the location of the binding site, we have proposed to divide this family into three subfamily groups (Bockaert and Pin, 1999).

Subfamily 1a contains GPCRs for small ligands such as rhodopsin, catecholamines, odorants, small peptides, ATP etc…. the binding site of agonists is localized inside the 7 TMs at a distance of about 10Å (Palczewski *et al.,* 2000; Tota and Strader, 1990).

The sub-family 1b includes most GPCRs for peptides in which the binding site is localized at the external face of the receptors, including the N-terminal, the extracellular loops and the TM domains at the frontier of the extracellular surface (Bockaert and Pin, 1999).

Family 1c includes GPCRs for glycoproteins such as TSH, LH etc… The binding site is essentially extracellular, within the N-terminal domain and made by a structure containing a series of leucine-rich repeats forming a crescent with the concave inner surface, consisting of β-sheets which may bind ligands (Kajava *et al.,* 1995; Phang *et al.,* 1998).

The intracellular loops contain the classical three loops and at least in rhodopsin, but likely in many other GPCRs, a fourth loop (C-IV) (figures 1A and 2D) made of an α-helical structure after TM-VII and before the cysteines of the C-terminal which are palmitoylated. The corresponding peptides are reported to prevent rhodopsin from activating the G-protein, transducin (Gt) (Palczewski *et al.*, 2000). Such an α-helical structure in this C-IV region has been demonstrated in turkey β-adrenergic receptors by solution NMR spectroscopy (Jung *et al.*, 1996). This α-helical structure is amphiphilic, as is the C-terminal domain of C-III. Both regions are likely to be very important for the specificity and the potency of GPCRs to activate G-proteins. The C-II and C-IV project laterally from either side of the receptor creating a platform of 43Å that may be broad enough to allow rhodopsin to interact simultaneously with the α and γ subunits of G-proteins (Bourne and Meng, 2000) (figure 2C).

Several GPCRs from this family have particularly interesting structural or functional properties:

Protease activated GPCRs (PARs) are activated when thrombin (PAR1, PAR3, PAR4 receptors) or trypsin and tryptase (PAR2 and PAR4) binds to and cleaves its amino-terminal exo-domain to unmask a new receptor amino-terminus. This new amino terminus then serves as a tethered peptide agonist ligand, binding intra-molecularly to the body of the receptor (Coughlin, 1999).

In a number of herpes and pox viruses, genes coding for homologues of chemokines, as well as chemokine GPCRs (Wells and Schwartz, 1997) have been described. The virus chemokine ligands are antagonists used to prevent the local recruitment of leukocytes. The function of virus GPCRs is less clear, some of them are constitutively active (see section VII) and may be implicated in malignancies such as Kaposi's sarcoma or acceleration of vascular diseases (Geras-Raaka *et al.*, 1998).

The entry of the human immuno-deficiency virus (HIV) into human CD4-positive cells depend on the presence of both CD4 receptors and a GPCR which is generally CXCR4 (natural ligand, SDF-1 – stromal derived factor 1) in T-tropic HIV-1 strains and CCR5 (natural ligands RANTES, MIP-1α, MIP-1β - macrophage inflammatory protein 1 alpha) in M-tropic strains. T-tropic strains infect primary T lymphocytes and appear at the late stage of the disease, whereas M-tropic strains infect monocytes / macrophages and have been shown to be responsible for viral transmission (Berger *et al.*, 1999). The mechanism of HIV entry involves the interaction of the envelope protein gp160 (cleaved to gp120 and gp41 by the cell) with CD4, the association of gp120 with GPCR and finally the dissociation of gp41 from gp120 and its participation in the virus fusion. A mutant allele of the CCR5 gene, Δ32 ccr5 found in Caucasian populations was shown to provide homozygotes with a strong resistance to infection by HIV (Samson *et al.*, 1996). A north to south gradient was found with the highest allele frequency in Finland and Mordvinia (16%) and the lowest in Sardinia (4%). Data indicates that most ccr5 alleles originate from a single mutation event that took place a few thousand years ago in Northeastern Europe. The high frequency suggests that a selection advantage is associated with this mutation (Libert *et al.*, 1998). Note that the CCR5 variants account for a small proportion of individuals with apparent HIV resistance. In addition, a structural variant of the chemokine receptors CX3CR1 appears to confer a more rapid progression of the pathology (Faure *et al.*, 2000). Drugs interfering with CCR5 and CXCR4 are actively researched and constitute a hope in the treatment of this infection.

Family 2: this family includes the smallest number of GPCRs (figure 3) including those for secretin, calcitonin, CRF (corticotropin releasing factor), VIP (vasoactive intestinal peptide), PACAP (pituitary adenylate cyclase activating polypeptide) and glucagon. The binding site is mostly extracellular, but E loops and part of the TMs exposed to extracellular domains are likely to be involved. Calcitonin and PACAP receptor N-terminal splice variants have been

described to have a modified pharmacology (Houssami *et al.,* 1994; Pantaloni *et al.,* 1996). Note that we have included a series of receptors within this family (although figure 3 indicates that they are structurally closed, but distinct from the classical list of hormonal receptors quoted above) which belong to LNB-TM7 (see introduction). This is the case for the latrotoxin receptor, a receptor for the toxin of the spider called "Black widow" known to strongly stimulate neurotransmitter release in a Ca^{2+}-independent manner (Davletov *et al.,* 1998; Krasnoperov *et al.,* 1997). Also included in this subgroup is EMR1, a LNB-TM7 protein localized in macrophages, BAI-1 and also BAI-2 and BAI-3 are LNB-TM7 proteins exclusively expressed in brain (Stacey *et al.,* 2000).

Family 3: the prototype of this family are the metabotropic glutamate receptors (mGluRs). The first one to be described was coupled to phosphoplipase C (Nicoletti *et al.,* 1986; Sladeczek *et al.,* 1985). Now eight mGluRs have been cloned and classified into three groups, group I (mGluR1 and 5) coupled to PLC, group II (mGluR2 and 3 and a Drosophila receptor DmGluRA - Parmentier *et al.,* 1996) and group III (mGluR4, -6, -8). An N-terminal truncated mGluR is proposed to be a putative glutamate taste receptor (Chaudhari *et al.,* 2000) negatively coupled to adenylyl cyclase (Pin *et al.,* 1999; Pin and Duvoisin, 1995). The signaling properties of mGluRs are far more complex (Fagni *et al.,* 2000). Family 3 now includes: calcium-sensing receptors (CASR), a specific family of pheromones (VR) (reviewed in Bargmann, 1997), putative vertebrate taste receptors (TR) (Hoon *et al.,* 1999), $GABA_BR1$ (Kaupmann *et al.,* 1997) and $GABA_BR2$ receptors (Jones *et al.,* 1998a; Kaupmann *et al.,* 1998; Kuner *et al.,* 1999; Ng *et al.,* 1999; White *et al.,* 1998), and goldfish basic amino-acids receptor (Speca *et al.,* 1999). Closed, but slightly distinct, is the bride of seven-less (BOSS), which is a ligand of seven-less tyrosine kinases involved in eye differentiation in Drosophila (Hart *et al.,* 1990). However, the fact that a G-protein is required for its action has not yet been demonstrated.

Family 3 GPCRs possess a typical but unique feature, being that they have a large extracellular domain that shares some sequence similarities with bacterial periplasmic amino acid-binding proteins (PBPs), such as the leucine / isoleucine / valine-binding protein (LIVBP) (O'Hara *et al.*, 1993) and acetamide-binding protein (Amic-C) (Bessis *et al.*, 2000). These proteins are characterized by a bilobate structure with ligand binding in a cleft which can open or close like a Venus flytrap. The extracellular domain of mGluR1, 4 and $GABA_B1$ in isolation, is enough to bind their ligands (Malitschek *et al.*, 1999; Okamoto *et al.*, 1998), a conclusion supported by the possibility of exchanging the extracellular domains of some of them keeping the entire pharmacology associated (Parmentier *et al.*, 2000a; Pin *et al.*, 1999) and by the recent crystallization of extracellular domains of mGluR1 associated or not with glutamate (Kunishima *et al.*, 2000).

Mutagenesis of both mGluRs (Pin *et al.*, 1999), $GABA_B1$ (Galvez *et al.*, 2000a; Galvez *et al.*, 2000b), and CASR as well as crystal structures of mGluR1 (Kunishima *et al.*, 2000), modelization of mGluRs (Bessis *et al.*, 2000) and $GABA_BR$ (Galvez *et al.*, 2000a), give a clear picture of the binding sites.

Kunishima et al. (Kunishima *et al.*, 2000) determined the structure of two mGluR1 un-liganded forms and a complex form with glutamate (figure 4). All crystals contain two protomers (dimer) connected via a disulfide bridge between Cys^{140}. The dimerization of GPCRs will be the subject of section IV. The N-terminal binding domain is made of two lobes each made of two domains separated by three linkers (L 1-3) in the following order on the primary sequence and starting from the N-terminus: LB1a, L1, LB2a, L2, LB1b, L3, LB2b, followed by a cysteine-rich (CR) domain not present in $GABA_BRs$. The binding site is indeed localized within the cleft at the surface of lobe 1a (LB1a) in the open state and the bound ligand also contacts with lobe 2 (LB2a) in the closed state. Each domain can adopt an open or closed state and in addition, the two protomers in the dimer can adopt two positions called

"active = A" and "resting = R", differing in the orientation of an α-helical interface. The two un-liganded crystallized forms correspond to a closed-open /A and an open-open /R form respectively, whereas the liganded form corresponded to only one of them, the closed-open/A. There was no difference in the latter due to the presence of glutamate. This confirms previous thoughts proposing that agonists stabilize rather than induce active forms. The feasibility of other putative conformers has been demonstrated such as closed-open/R, closed-closed/R, open-open/A and closed-closed/A (Kunishima *et al.*, 2000). In the open state, glutamate binds to two residues conserved in the entire mGluR family including Drosophila and *Caenorhabditis elegans*. These residues are Ser^{165} and Thr^{188} in mGluR1 which form hydrogen bonds with the α-carboxyl and the α-amino-group, respectively. The γ-carboxyl is linked to a conserved Arg residue (Arg^{78} in mGluR1) via a H_2O molecule and to Tyr^{74} via a hydrogen bond (Kunishima *et al.*, 2000). Additional contacts exist in the open, as well as in the closed state (Kunishima *et al.*, 2000).

Mutagenesis and modeling of $GABA_BR1$ binding sites indicate that the carboxyl of GABA (equivalent of the γ-carboxyl of glutamate) binds to Ser^{246} (homologue of Ser^{165} of mGluR1 which by the α-carboxyl) in LB1a, whereas its amino group binds Asp^{471} in LB1b (Galvez *et al.*, 2000a). Ser^{269} (homologue of $Thre^{188}$ in mGluR1) is necessary for the potentiation of $GABA_BR1$ activation by Ca^{2+}, suggesting that Ca^{2+} can compensate the lack of the α-amino group of GABA (Galvez *et al.*, 2000b).

Family 4: this is another family of putative pheromone receptors (VN1,-6) cloned from sensory Gαo neurons of the vomeronasal organ which co-express the Gαi protein. This is in contrast to the observation that VRs from family three are expressed in vomeronasal neurons, which also express Gαo (Bargmann, 1997).

Family 5: the frizzled receptor family (11 members identified in mice) which are either activated or inhibited by Wnt proteins (the name is derived from mouse Int-1 and Drosophila

Wingless) and control embryonic development, cell proliferation and determination (Patapoutian and Reichardt, 2000; Perrimon, 1996). These receptors activate two down-stream signaling pathways; one controls gene transcription and the other G-protein mediated Ca^{2+} influx. The immediate proteins activated by frizzled receptors are unknown, but the important APC (adenomatous polyposis coli) protein, often mutated in colorectal cancer, lies in its pathway. This family also contains receptors for “smooth” which are interesting because they appear to be under a permanent negative control of “patched”, a 10 TMs receptor for the morpho-genetic factor “hedgehog”. The binding of hedgehog to patched releases this inhibition (Perrimon, 1996; Ruiz i Altaba, 1997). MOM-5 and LIN-17, are *Caenorhabditis elegans* GPCRs, the latter required for certain asymmetric cell divisions.

Family 6: this family of cAMP receptors has only been described in the slime mold called *Dictyostelium discoideum* and are implicated in chemo-attraction (Kim *et al.*, 1998; Klein *et al.*, 1988).

Family 7: this family are taste receptors (T2R) of vertebrates and mediate bitter taste via activation of gustducin, a G-protein which activates cGMP phosphodiesterase and homologue to transducin (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000).

Family 8: this is the family for odorant Drosophila olfactory receptors (DOR) localized in antenna and maxillary palp (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999).

In conclusion to this section on the diversity of GPCRs and to follow the idea that evolution has "tinkered" GPCRs to adapt their structure to a wide range of ligands having very different structures (Bockaert and Pin, 1999), we can notice that genes coding for 7 TM proteins may have fused with genes coding for binding domains of other proteins to generate genes coding for new families of GPCRs. The same binding domains may also have fused with one TM protein to generate other receptors. Such a situation is found in glycoprotein hormone GPCRs in which the extracellular binding site is composed of several leucine repeats, similar to those

found in Toll receptors (one TM receptor) implicated in inflammation (see Hsu *et al.,* 1998; Kajava *et al.,* 1995). Secretin and frizzled receptors both share a cysteine-rich extracellular amino terminus that is thought to be involved in ligand binding and which is conserved in diverse proteins including several one TM receptor tyrosine kinases such as those of the Ror family or the MusSK family (Xu and Nusse, 1998). Furthermore, as already discussed, the binding site of family 3 is homologous to the binding site of ionotropic glutamate receptors (Armstrong *et al.,* 1998) Ami-C (Bessis *et al.*, 2000) and atrial natriuretic peptide/guanylate cyclase receptors and procaryote proteins such as PBPs (see http://www.pharmsci.org/, volume 1, issue 2).

IV. Homo- and hetero-dimerization of GPCRs, and interaction with other TM proteins: a revolution in current concepts of GPCR structures and functions

For many years, the classical view of GPCR / G-protein coupling stoichiometry was thought to be one receptor for one G-protein. However, there was some evidence in favor of the formation of dimers and even oligomers. Experimental evidence was provided in the early 80's by several groups, with the use of radiation inactivation, which indicated that GPCRs, G-proteins and AC acted as functional units that were larger than predicted for simple monomeric structures (Fraser and Venter, 1982; Salahpour *et al.,* 2000; Schlegel *et al.,* 1979). This was modeled by M. Rodbell in the early 90's (Rodbell, 1995). In addition, it was not conceivable that binding of ligands on the extracellular domain, like the one present in family 3 GPCRs, could modify the structure of the TM domains and intracellular loops without using dimerization or oligomerization, in order to induce an allosteric change in the whole structure. This situation is similar to that demonstrated in tyrosine kinase receptors (Changeux and Edelstein, 1998).

A. Homo-dimerization of GPCRs

The recent interest in dimerization of GPCRs arose from functional studies in which inactive point-mutated (angiotensin AT1) (Monnot *et al.,* 1996) or chimeric receptors (α2-AR - m3 muscarinic composed of the first five TM domains of one receptor and the last two TM domains of the other receptor), were not functional and did not bind ligands, but recovered these properties when co-expressed (Maggio *et al.,* 1993). In the case of the chimeric receptors, both α2-AR binding and m3 binding as well as coupling were restored which indicates that the inter-dimerization restored two "correct" binding pockets. Similarly, co-expression of CASRs that harbor inactivating mutations in distinct domains, could also partially rescue Ca^{2+} responses (Bai *et al.,* 1999). In contrast, some mutated and truncated GPCRs have been shown to behave as dominant negatives. This constitutes another indication of dimerization. In the case of truncated V2-vasopressin and CCR5 chemokine receptors, the dominant negative effect was due to intracellular retention (Benkirane *et al.,* 1997; Vila-Coro *et al.,* 2000; Zhu and Wess, 1998). This may indicate that dimerization is, at least for some GPCRs, required for trafficking to the membrane. This role of dimers for trafficking to the membrane has also been demonstrated for the $GABA_B$ heterodimer (Margeta-Mitrovic *et al.,* 2000). In addition, the dominant negative effect of CCR5Δ32 has been proposed to be the reason for the slow onset of the disease observed in AIDS patients who are heterozygotes for this mutation (Benkirane *et al.,* 1997; Vila-Coro *et al.,* 2000).

In addition to functional studies, co-immunoprecipitation of differentially epitope-tagged receptors has allowed a great number of studies to demonstrate the reality of the dimerization. More interesting, dimerization has been demonstrated *in vivo* (*in cellulo*) using FRET (fluorescence resonance energy transfer) in the case of alpha-mating factor receptor in living yeast (Overton and Blumer, 2000), or somatostatin receptors (Rocheville *et al.,* 2000b) and

BRET (bioluminescence resonance energy transfer) in the case of β2-AR (Angers *et al.*, 2000).

In total, dimerization has been found so far in the following receptors:

- family 1 GPCRs: β2-adrenergic (Herbert *et al.*, 1996), angiotensin AT1 (Monnot *et al.*, 1996), δ-opioid (Cvejic and Devi, 1997), m3 muscarinic, somatostatin (Rocheville *et al.*, 2000b), vasopressin V2 (Schulz *et al.*, 2000), dopamine D1 (George *et al.*, 1998), bradykinin-B2 (Quitterer *et al.*, 1999);
- family 3 GPCRs: mGluR1 and mGluR 5 (Kunishima *et al.*, 2000; Okamoto *et al.*, 1998; Romano *et al.*, 1996; Tadokoro *et al.*, 1999; Tsuji *et al.*, 2000), and CASR (Bai *et al.*, 1998; Goldsmith *et al.*, 1999);
- α-mating factor GPCR (Overton and Blumer, 2000).

This is not an exhaustive list.

B. Hetero-dimerization of GPCRs

The first surprising observation that GPCRs can form heterodimers came from family 3. Cloning of $GABA_B$ receptors revealed that they are composed of two non-functional subunits ($GABA_BR1$ and $GABA_BR2$) sharing sequence similarity. Co-expression of $GABA_BR1$ and $GABA_BR2$ gave rise to a functional receptor, efficiently coupled to G-proteins (Jones *et al.*, 1998a; Kaupmann *et al.*, 1998; Kuner *et al.*, 1999; White *et al.*, 1998). Recently, more surprises came from the observations that somatostatin and dopamine D2 GPCRs (Rocheville *et al.*, 2000a), κ and δ (Jordan and Devi, 1999), as well as μ and δ opioid receptors (George *et al.*, 2000), angiotensin AT1 and bradykinin-B2 (AbdAlla *et al.*, 2000) can heterodimerize.

C. Structure of homo- and heterodimers

The diversity of solutions selected during evolution to form homo- and heterodimers is quite surprising, but may indicate that formation of such dimers is crucial for generating diversity of coupling and pharmacology.

Homo-dimerization via the N-terminal domain is found in the following receptors: bradykinin-B2 receptors (AbdAlla *et al.,* 1999), mGluR1,5 (Kunishima *et al.*, 2000; Okamoto *et al.*, 1998; Romano *et al.*, 1996) and CASR (Goldsmith *et al.,* 1999). In the case of CASR and mGluRs, dimerization occurs via one conserved Cys residue localized on lobe I of the LIVBP binding site (Goldsmith *et al.,* 1999; Kunishima *et al.*, 2000; Romano *et al.*, 1996; Tsuji *et al.,* 2000). The S-S bond is not the only mechanism of dimerization (Robbins *et al.*, 1999). This bond certainly functions as an inter-promoter linker to increase the concentration of dimers (Kunishima *et al.*, 2000). Dimerization has also been proposed to occur via the highly conserved Cys residues of E2 and E3 was found in m3 muscarinic receptors (Zeng and Wess, 1999).

Dimerization via TM regions has been suggested, following observations that peptides corresponding to sequences present in these regions and especially those from TM-VI and TM-VII, disturb dimerization and sometimes functions of β2-adrenergic (Hebert *et al.,* 1996), D2 dopaminergic (Ng *et al.*, 1996), vasopressin V2 (Schulz *et al.*, 2000) and CXCR4 receptors (Tarasova *et al.,* 1999).

Homo and hetero-dimerization via the C-terminal tail has been reported for δ-opioid receptors (Cvejic and Devi, 1997) and $GABA_BR1$ and $GABA_BR2$ receptors. In the latter, the interaction occurs via a coiled-coil α-helical structure (Kammerer *et al.,* 1999; Kuner *et al.,* 1999).

D. Functional roles of homo-dimerization

The first question is to find out whether agonists induce or increase dimerization. The answer is not clear, agonists either do not modify dimerization (α-mating receptors - Overton and Blumer, 2000), slightly induce dimerization (β2-AR - Angers *et al.*, 2000), potently induce dimerization (bradykinin B2 receptors - AbdAlla *et al.*, 1999); somatostatin receptors - Rocheville *et al.*, 2000b) or even disrupt pre-existing dimerization (δ-opioid receptors - Cvejic and Devi, 1997). The necessity for homo-dimerization for a correct trafficking to the plasma membrane has already been discussed and is likely (Benkirane *et al.*, 1997; Vila-Coro *et al.*, 2000; Zhu and Wess, 1998). The absolute necessity for homo-dimerization for coupling to G-proteins has not been clearly demonstrated (Rocheville *et al.*, 2000b) (see however δ-opioid receptors - Cvejic and Devi, 1997).

E. Functional roles of hetero-dimerization

Before discussing the functional role of hetero-dimerization, evidence for such associations in naturally-existing tissues has to be discussed imperatively. In the case of $GABA_B$ receptors, extensive co-localization of $GABA_BR1$ and $GABA_BR2$ subunits (except in striatum) favors such naturally-occurring dimers (Kaupmann *et al.*, 1998). Similarly, somatostatin and dopamine D2 receptors are co-localized in brain cortex and striatum (Rocheville *et al.*, 2000a). An elegant experiment performed in smooth muscle cells indicates that antisense knock-out of bradykinin B2 receptors inhibit the angiotensin AT1 response (AbdAlla *et al.*, 2000). The role of $GABA_BR1$ hetero-dimerization is now quite clear. Alone, the $GABA_BR2$ subunit, which is inactive, is required for cell surface expression of the $GABA_BR1$ subunit (Jones *et al.*, 1998b; Kaupmann *et al.*, 1998; Kuner *et al.*, 1999; Margeta-Mitrovic *et al.*, 2000; White *et al.*, 1998). Indeed, $GABA_BR1$ is retained in the endoplasmic reticulum through a C-terminal retention / retrieval motif Arg-Lys-Arg (RKR), reminiscent of a similar

sequence in ATP-sensitive K^+ channels. Interaction of $GABA_BR1$ and $GABA_BR2$ through their C-terminal coiled-coil α helical structure, masks the retention signal in $GABA_BR1$ (Margeta-Mitrovic *et al.*, 2000). Moreover, Galvez et al. have recently shown that the central core and intracellular domains of $GABA_BR2$ contain the molecular determinants required for recognition and activation of the proteins. The central core and intracellular domains of $GABA_BR1$ improved coupling efficacy (Galvez *et al.*, 2001). In terms of pharmacology, hetero-dimerization may be a fine-tuning mechanism to increase the pharmacological diversity and coupling efficiency. In the AT1-bradykinin B2 dimer, the coupling of AT1 to messenger production is greatly increased by the presence of a functional or even a non-functional bradykinin-B2 receptor (AbdAlla *et al.*, 2000). The pharmacology of the κ−δ opioid receptor dimers and μ−δ receptor dimers are obviously different from those of the corresponding homo-dimeric corresponding receptors (George *et al.*, 2000; Jordan and Devi, 1999). This is, of course, interesting for drug development, but at the same time, quite puzzling.

F. Hetero-dimerization of GPCRs with receptor channels

The curiosity of some researchers for, strange and up to now, unbelievable associations, led Liu et al. to look for a direct protein-protein coupling between dopamine D5 (a Gs coupled GPCR) and $GABA_A$ ionotropic receptors (Liu *et al.*, 2000). Indeed, both receptors are co-localized in dendritic shafts and the cell soma/axon hillock area where inhibitory GABAergic neurons form major post-synaptic contacts. These authors showed that the C-terminus of D5 receptors (but not D1 receptors), selectively binds the second intracellular loop of the GABAγ2 receptor subunit. This physical association enables a mutually inhibitory functional interaction between these receptors. The physiological and therapeutic consequences of such an *a priori* bizarre marriage, remain to be demonstrated.

G. Hetero-dimerization of GPCRs with one TM protein

Nina A from *Drosophila melanogaster* and its vertebrate homologue RanBP2, two cyclophilin-related proteins, have been known for some years to bind opsins for folding and transport (Baker *et al.*, 1994; Ferreira *et al.*, 1996). In *Caenorhabditis elegans*, odr-4 and odr-8 are required to localize a subset of odorant GPCRs to cilia of olfactory neurons (Dwyer *et al.*, 1998). More surprising and disturbing for pharmacologists, is the report that the CRLR (calcitonin receptor-like receptor) is a virtual receptor which generates the CGRP (calcitonin gene-related peptide) receptor when associated with RAMP1 (receptor activity-modifying protein) and the adreno-medullin receptor when associated with RAMP2. RAMP1 and 2 are also required for a correct glycosylation and transport of CRLR to plasma membranes.

A yeast two-hybrid screen with the C-terminal 81 residues of the D1 receptor used as bait, allowed Lezcano et al. (Lezcano *et al.*, 2000) to fish out a one-TM protein coding clone of the P19/21 calcyon family. Calcyon and dopamine D1 GPCRs are co-expressed in macaque brain within the spines of the CA3 region of hippocampus and caudate nucleus. Interestingly, D1 coupling to Gs is shifted to Gq when co-expressed with calcyon. In brain and kidney, D1 agonists have also be found to increase 1,4,5-trisphosphate turnover (see refs in Lezcano *et al.*, 2000).

V. Activation of GPCRs and G-proteins

The structures of the different families of GPCRs are so diverse, that their mechanisms of activation are also likely to be quite different, although a common fundamental mechanism may exist at the final step.

A. Activation of the central core of GPCRs

The crucial observations that single mutations localized in intracellular loops, but also within the TMs or even in the extracellular loops or domains, may lead to constitutively active receptors, will be discussed in more detail in section VII. These data are in favor of an allosteric model in which constrained receptors R loses some intra-molecular constraints and undergo a conversion to an active state R*. We have already described some of these constraints in section III (figure 5). In rhodopsin, a salt bridge exists between Lys^{296} of TM-VII (the retinal attachment site) and E^{113} in TM-III (the Schiff base counterion) (Palczewski *et al.*, 2000; Robinson *et al.*, 1992) as well as: (1) an interaction between Asn^{73} of TM-I and Tyr of the conserved motif NPXXY in TM-VII; (2) interactions between Asn^{55} in TM-I, Asp^{83} in TM-II and Ala^{299} in TM-VII, as well as Asn^{302} (of NPXXY) via a water molecule (the so-called “polar pocket”, also described in α1-AR (Scheer *et al.*, 1996) as described by (Palczewski *et al.*, 2000). This “polar pocket" is well conserved in family 1. In α1b-AR, a salt bridge between Asp^{125} of TM-III, and Lys of TM-VII, constrains the receptor in the R state (Porter and Perez, 1999). Similarly, in AT-1 receptor stabilization of TM-III and TM-VII, via Asn^{111} of TM-III and Tyr^{292} of TM-VII, has been described (Groblewski *et al.*, 1997). In C5a-R, many mutations of TM-III and TM-VI lead to constitutive activation. Some of them suggest that hydrophobic constrains occur for example between Ile^{124}/Leu^{127} of TM-III and Phe^{251} in TM-VI (figure 5) (Baranski *et al.*, 1999). Other constraints are evident in the rhodopsin crystal (Palczewski *et al.*, 2000) and it is likely that each receptor has its own. How these constraints are released and how agonists stabilize the active forms, remain to be elucidated. One key event is certainly a change in the environmental situation of the DRY (or ERY) sequence and the resulting protonation of the aspartic / glutamic acid (Arnis *et al.*, 1994; Oliviera *et al.*, 1994; Scheer *et al.*, 1996; Scheer *et al.*, 1997). This protonation has been directly measured in rhodopsin (Arnis *et al.*, 1994). An indirect argument in favor of this

hypothesis is the fact that charged-neutralizing mutations which mimic the non-protonated state of the aspartic acid, cause dramatic constitutive activation of α1b, vasopressin and rhodopsin GPCRs (Cohen *et al.,* 1993; Morin *et al.,* 1998; Scheer *et al.,* 1996). The role of the arginine of the DRY sequence is crucial. Its mutation impairs the response mediated by GPCRs linked to different signaling pathways ((Scheer *et al.,* 2000) and discussion within). Two hypotheses have been proposed to define the specific role of aspartate / arginine of DRY. In the first one, arginine is constrained in the R state, in the "polar pocket", causing an ionic interaction with the conserved aspartate in TM-II (Scheer *et al.,* 1996). Following activation and protonation of the aspartate of DRY, molecular modeling indicates that the arginine breaks its interaction with the aspartate of TM-II and shifts out of the pocket. In the second hypothesis, based on computational simulations in GnRH receptors, it has been suggested that, in the "inactive state", the ionic counterpart of the arginine of DRY is the adjacent aspartate (or glutamate) (Ballesteros *et al.,* 1998). Upon activation, this ionic interaction is broken and the arginine of DRY now induces ionic interaction with the aspartate of TM-II. In favor of the latter hypothesis is the fact that mutation of the aspartate of TM-II disturbs functional coupling (see (Gether, 2000) and that in rhodopsin, the aspartate of TM-II is more strongly hydrogen-bound upon activation (Rath *et al.,* 1993).

The physical modifications of the central core of family 1 and 2 GPCRs are far from being understood, but some data have proven to be interesting. The main message is that the intra-molecular constraints between the TMs, described above, are released. This is generally associated with thermal instability (Claeysen *et al.,* 2001; Gether *et al.,* 1997a; Rasmussen *et al.,* 1999) of the receptor and a separation of the TMs, leading to an increase in the crevice surface of the receptor forming contacts with G-proteins (figure 5). Displacements have been observed between TM-III and TM-VI. Using the cysteine accessibility method, Javitch et al. described that a cysteine in TM-VI (Cys^{285}), inaccessible to a hydrophilic sulfhydryl-reactive

reagent in the R state, becomes accessible in constitutively active β2-AR (Javitch *et al.*, 1997). This indicates a clock-wise rotation (looking at the receptor from the cytoplasmic surface) of TM-VI. Using tryptophan UV-absorbance spectroscopy, Lin and Sakman obtained direct evidence that rhodopsin activation involves relative movements of TM-III and TM-VI (Lin and Sakmar, 1996). Site-directed labeling of single cysteines, inserted at the cytoplasmic side of the transmembrane helical structures (Cys^{139} in TM-III and Cys^{285}, Cys^{248} in TM-VI) with sulfhydryl-specific nitroxide spin labels, provides evidence for clock-wise (30°) and separation movements of TM-III and TM-VI (Farrens *et al.*, 1996). A recent cross-linking experiment indicates that the retinal ionone is attached to Trp^{265} in TM-VI, just as predicted in the crystal structure in the R state (Borhan *et al.*, 2000). In the R* state, the ionone ring is cross-linked to Ala^{169} of TM-IV. Bourne and Meng have pointed out that if the *trans*-retinal interacts in such a way, this implies considerable movement of TM-III, but also movement of TM-IV and VII (Bourne and Meng, 2000). Similarly, in β2-AR, fluorescent labeling of cysteines in TM-III and TM-VI has provided a way of measuring an increase in fluorescence (accessibility to a more polar environment), upon agonist activation (Gether *et al.*, 1997b). Favoring the importance of the TM-III and TM-VI movements upon activation, is the fact that binding of Zn^{2+} to the bis-His metal ion binding site, constructed between these TMs, blocks rhodopsin, β2-AR and PTH receptor activation (Sheikh *et al.*, 1999; Sheikh *et al.*, 1996). There is certainly some variability in the mechanisms by which the different families activate the central core. In family 1c, glycoprotein binding to the N-terminal part is likely to generate a special way of activation. G. Vassart's group (unpublished data) has recently proposed that the N-terminal domain constrains the central core. This constraint would be released by the glycoprotein hormones. In the case of family 3, the twist between the two protomers of the dimer (see section III), has been proposed to be associated with a relative movement of their central cores leading to activation.

In the section IV dealing with the dimerization of GPCRs, we have clearly shown that homo- or hetero-dimerization of family 3 GPCRs is required for their activation. In family 1 GPCRs, the role of dimerization in activation is not quite as clear.

The platform formed by the intracellular loops, including intracellular loop C-IV (43Å), is sufficient for simultaneous interaction with the carboxyl terminal of Gα and Gγ (Bourne and Meng, 2000).

B. Intimate contacts between GPCRs and G-proteins

Numerous studies have been dedicated to the research of GPCR domains that may confer specificity of interaction with particular G-proteins. The general idea is that most intracellular domains, including the C-IV loop and the C-terminal domain, are implicated, whereas no consensus sequences have been recognized. It is possible that each receptor has designed its own solution. For two very close receptors like vasopressin V2 (coupling to Gs) and V1a (coupling to Gq), the specificity of interaction is localized in C-I and C-III loops, respectively (Liu and Wess, 1996). In many family 1 GPCRs, the C-terminal part of the C-III loop determines the specificity. When four VTIL residues of the VTRTIL sequence, localized in the C-terminal of C-III of muscarinic m2 receptors (coupled to Gi), are introduced in the homologous positions of the muscarinic m3 receptor (coupled to Gq), the chimera is now able to activate Gi (Liu *et al.*, 1995). These four residues are likely to be on the same face of an α-helix. There are six splice variants of PACAP receptors differing within the C-terminal part of the C-III loop, by introduction of "hip" and "hop" cassettes. PACAP receptors can be coupled to both AC and PLC. The presence of the hip cassette suppresses the PLC coupling (Spengler *et al.*, 1993). Mutations of the key residue in the C-III intracellular loop often results in constitutive activation (Claeysen *et al.*, 2000; Kjelsberg *et al.*, 1992) (see section VII). In mGluRs, the C-II loop and the putative C-IV intracellular loop, determine the specificity of

coupling to G-proteins (Gomeza *et al.,* 1996a; Gomeza *et al.,* 1996b; Mary *et al.,* 1998; Parmentier *et al.,* 1998; Prézeau *et al.,* 1996). Editing the 5-HT_{2C} mRNA within the sequence coding for the C-II loop, leads to proteins with different coupling signaling characteristics (Burns *et al.,* 1997). Finally, phosphorylation of different intracellular domains induces a fine-tuning of the coupling to G-proteins, including desensitization and change from Gs to Gi coupling (Daaka *et al.,* 1997) (section VIII). The capacity of mGluR5 to produce an oscillatory intracellular Ca^{2+} release, depends on PKC phosphorylation of a specific threonine residue of the C-terminal domain (Kawabata *et al.,* 1996).

An important domain of Gα, implicated in the interaction and the specificity of a given GPCR to a given G-protein, concerns the last few residues of their C-terminus (figure 2). The residue (-4) of Gαi, Gαo and Gαt is a cysteine residue that is ADP-ribosylated by Pertussis toxin, a covalent modification that prevents the interaction of G-proteins with receptors (Hamm, 1998). In the Gq family, residue -4 is a tyrosine that has to be phosphorylated for an efficient coupling to PLC-activating receptors (Umemori *et al.,* 1997). Gαq chimeras, in which the last 4-5 residues of Gq have been replaced by those of Gαi or Gαo, are still coupled to PLC, but are activated by receptors naturally coupled to Gαi or Gαo, such as dopamine D2, muscarinic m2, mGluR2, 3, 4 and 8. Interestingly, there are no sequence similarities at all between receptors from different families (Blahos *et al.,* 1998; Conklin *et al.,* 1993). The most important residue for the specificity of interaction is α4 in Gαi and Gαo, whereas two residues are important for the specificity of interaction with Gαq (Kostenis *et al.,* 1997). Two other domains of Gα are important for the coupling, as well as for its specificity (figure 2). These are the α4 and α5 helices, plus the L9 and β6 on the one hand (Mazzoni and Hamm, 1996; Noel *et al.,* 1993) and a short region between the N-terminal α-helix and β1 strand, on the other (Blahos II *et al.,* 2001). A photoactivated peptide derived from the C-III loop of α2-

AR, is cross-linked with the N terminal domain of Gαo, and also with the C-terminal part of Gβ. Rhodopsin, as well as β2-AR, bind to Gβ (Taylor *et al.*, 1994; Taylor *et al.*, 1996).
The scenario leading from these interactions to the change in the conformation of Gα, especially at the level of switches I, II and III, followed by the release of GTP (figure 2) is not known. H. Bourne has proposed two routes; one via α5-β6, a region in which a mutation induces rapid GDP release, the other via a change in the orientation of Gα and Gβ, due to the interaction of receptor intracellular loops, with the cavity separating these two subunits (Bourne, 1997b).

VI. Orphan GPCRs

The current explosion of genomic sequence data provides a way to identify many more GPCR members in human and other species, genomes. Most, if not all newly identified GPCRs, falls into the category of orphan receptors for which the endogenous ligand remains to be identified. Typically, these GPCRs show only low levels of similarity (less than 35% sequence similarity) with known GPCRs. For family 1, more than 80 orphan GPCRs are known (Lee *et al.*, 2001).
The rationale for investing resources in characterizing orphan GPCRs is certainly to increase our fundamental knowledge, although this is no longer a pertinent driving force. A stronger motivation is the fact that GPCRs have a proven history of being excellent, directly or indirectly (anti-depressant drugs for example), targets of the majority of drugs (Wilson *et al.*, 1998). Another reason is that there is an increasing number of diseases associated with GPCR mutations (see section IX). GPCR mutations and polymorphism are likely to be associated with pathologies and/or susceptibility to pathologies. The overall strategy has been called "reverse pharmacology". The research starts with extensive bio-informatical analysis of

expressed sequence tags (EST) databases. ESTs are short sequences representing transcribed genes and continuous protein coding regions. They can be used to localize the GPCR tissue expression by *in situ* hybridization. Full-length clones can be obtained and chromosomal localizations determined. A possible association with genetic diseases can be analyzed. Once the full-length clone is obtained, the receptor can be expressed in heterologous cells and functional assays can begin using a wide range of coupling mechanisms. The most popular assays are the measurement of changes in intracellular cAMP or Ca^{2+} levels either directly or through the use of reporter genes. The possibility of using promiscuous G-proteins, such as G15/G16 or chimeric Gqi, Gqo, Gqz, is widely used (Conklin *et al.*, 1993; Gomeza *et al.*, 1996b; Offermanns and Simon, 1995; Parmentier *et al.*, 1998). Once a functional assay is found, the search for the ligand can begin. Sometimes, a weak homology or a consensus sequence can be used to guess if the ligand for the orphan receptor is a peptide or a small ligand. Existing banks of compounds or biological extracts can be screened.

Every year, the natural ligands for several orphan receptors are discovered (Lee *et al.*, 2001). The following ligands have been discovered:

- nociceptin (orphanin) (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995);
- orexin (hypocretin) (Sakurai *et al.*, 1998);
- prolactin releasing hormone (Hinuma *et al.*, 1998);
- apelin (Tatemoto *et al.*, 1998);
- melanin-concentrating hormone (Chambers *et al.*, 1999; Saito *et al.*, 1999);
- urotensin (Ames *et al.*, 1999).

We are not going to provide an exhaustive review of all six, but we will take two recent examples.

Prolactin secretion is very closely controlled by many neurotransmitters. Only dopamine inhibits prolactin secretion, whereas many others, such as TSH and VIP, can stimulate

prolactin secretion. However, they were not really thought to be the most important physiological stimulators of prolactin receptors. Hinuma et al. (Hinuma *et al.*, 1999) first cloned an orphan GPCR called hGR3 which was largely expressed in the pituitary gland. Expression of this GPCR in cells and stimulation with hypothalamus bovine extracts led to the release of arachidonic acid. The use of this functional assay in combination with chromatographic fractionation of the extracts, led to the discovery of two peptides (PrP31 and PrP20) which stimulate the release of prolactin (Hinuma *et al.*, 1998). Hyperprolactinemia is a serious condition because this pathology inhibits reproduction. One can consider that mutations turning the hGR3 receptor to permanently active (see section VII), even in the absence of the PrP peptide, may be involved in the sterility of women suffering from hyperprolactinemia, especially those resistant to bromocryptine, a dopaminergic agonist which is the only drug available for this pathology. If this is the case, an inverse agonist may provide the solution.

The search for the ligand for an orphan receptor called HFGAN72, identified as an expressed sequence tag from human brain, was carried out in HPLC fractions of rat brain extract. The receptor was expressed in HEK cells and intracellular Ca^{2+} release was measured. This led to the discovery of two peptides termed "orexins -A and-B" after the Greek word "orexis" which means appetite (Sakurai *et al.*, 1998). Both peptides are produced from a single precursor, the prepro-orexin. Precursor and orexia peptides are localized in neurons of the lateral and posterior hypothalamus. The projections of these neurons are directed to the olfactory bulb, cerebral cortex, thalamus, and the midbrain region, including the locus coeruleus, raphe nucleus, and reticular formation. When administered centrally to rats, these peptides stimulate food consumption. Additionally, prepro-orexin mRNA level is up-regulated upon fasting, suggesting a physiological role for the peptides as central feedback mechanisms that regulate

feeding behavior. The dream story went on and the role of orexin receptors in narcolepsy was recently described (Lin *et al.*, 1999).

Narcolepsy is a disabling sleep disorder affecting humans and animals, characterized by daytime sleepiness, cataplexy, and striking transitions from wakefulness into rapid eye movement (REM) sleep. Lin et al. determined that canine Doberman (canarc-1 dogs) narcolepsy is caused by disruption of the orexin receptor 2 gene (Hcrtr2). In parallel, Chemelli et al. (Chemelli *et al.*, 1999) reported that, by behavioral and electroencephalographic criteria, orexin knockout mice exhibit a phenotype strikingly similar to human narcolepsy patients, as well as canarc-1 mutant dogs. Besides, Modafinil, an anti-narcoleptic drug with ill-defined mechanisms of action, was reported to activate orexin-containing neurons. Two recent reports indicate that in human narcolepsy, a reduction of orexin synthesis, rather than a mutation in its receptor, is involved (Peyron *et al.*, 2000; Thannickal *et al.*, 2000). Thus the pathway to therapy is now clear. In two years, the orphan receptor has led to the discovery of peptides implicated in appetite and sleep physiology and pathology… not too bad. There is no doubt that orphan receptors will lead to new and exciting discoveries over the next few years.

VII. Constitutive activity of allosteric GPCRs, inverse agonists and physiological relevance

A. Drug-receptor models

Until the 90's, pharmacologists working on GPCRs were still using the traditional “receptor occupation” theory that was the foundation of pharmacology for decades (Clark, 1933). The receptor was supposed to be "quiescent" under a R form in the absence of agonists (A). The agonist, when present, binds to R, inducing the formation of an AR complex (A+R ⇆ AR)

which triggers a cascade of events leading to physiological functions. The “ternary complex model” was introduced to account for the fact that GPCRs were found to bind agonists (not antagonists) with high and low affinity (De Lean *et al.,* 1980). This is because AR, but not R, is coupled with heterotrimeric G-proteins and induces the GDP-GTP exchange (figure 1B). The ternary complex AR-G (state 2 in figure 1B, G-protein empty) generally has a high affinity for the agonist, whereas AR and R generally have a lower affinity for the agonist. The antagonist has a unique affinity for R. The antagonist-R complex does not bind the G-protein. In 1965, Monod, Wyman and Changeux wrote a famous article entitled "On the nature of allosteric transitions: a plausible model" (Changeux and Edelstein, 1998; Monod *et al.,* 1965). The allosteric model proposed was based on observations made on bacterial enzymes that catalyze reactions of important biosynthetic pathways. They found that the catalytic activity of these enzymes can be regulated by the end product of the synthetic pathway which has no structural similarity to the substrate, and acts on a specific regulatory binding site, which is different from the catalytic site. The regulatory site induces a reversible alteration of the conformation of the protein: an allosteric transition. The observation that cooperative interactions exist for both the substrate and the ligand further extends the properties of allosteric proteins. The cooperativity of oxygen binding on the symmetrical tetramer hemes of hemoglobin distant by at least 25Å, further establishes the model. Several key features of allosteric proteins were first established for receptor channels, such as ionotropic receptors, nicotinic receptors and then GABA-benzodiazepine receptors (Changeux and Edelstein, 1998):

- the protein is an oligomer composed of several subunits and possesses one axis of rotation. As we have seen, GPCRs are probably associated as a dimer and possibly as oligomers, although the exact role of dimerization is not always clear except for $GABA_B$ receptors and mGluRs (Galvez *et al.,* 2001; Kunishima *et al.,* 2000) ;

- the oligomer can spontaneously exist in a minimum of two freely inter-convertible and discrete conformational states;
- the ligand binding stabilizes the particular state for which they exhibit a higher affinity;
- in the absence of agonists, the preexisting equilibrium between the inactive (R) and the active (R*) states is characterized by an isomerization constant J. Therefore, in the case of GPCRs we can write:

$$R \overset{J}{\leftrightarrows} R^* \text{ with } J = R/R^*.$$

This isomerization step is the foundation for the “extended ternary complex” (Samama *et al.*, 1993). Indeed, this explains the increased affinity of agonists and partial agonists for the constitutively active mutant receptors, in proportion to their efficacy, even in the absence of G-proteins (Samama *et al.*, 1993). In the “ternary extended model”, only R* and R*A can couple to G-proteins.

Several consequences were to be expected from such a model:

- depending on the value of J, GPCRs can exhibit constitutive activity, i.e. an activity in the absence of agonists, meaning that there is enough R* coupled to G to activate a detectable amount of effector activity;
- an antagonist is a drug which does not modify the equilibrium and therefore has an equal affinity for R and R*;
- an agonist is a drug with a higher affinity for R* than for R and which therefore shifts the equilibrium towards the R* form. The latter being the only one coupled to G-proteins in the extended ternary complex model which is the model we are presently examining and which was first proposed by Lefkowitz and his colleagues;

- ligands having a higher affinity for R than for R* should exist. In this case, they would displace the equilibrium towards R and reduce the constitutive activity, if any. These ligands are called "inverse agonists";
- depending on the relative affinity for R and R*, an agonist will be a full agonist or more or less partial.

Recently, Kenakin introduced the cubic model which differs from the ternary extended model by conveying the notion that R and R* can couple to G (and of course RA and R*A) (even though they have with different affinities). Thermodynamically, this is the most complete model (Kenakin and Weiss, 1996).

B. Inverse-agonists and constitutively active receptors

The existence of inverse agonists were first proposed by Braestrup et al. when they reported that DMCM (methyl 6,7-dimethoxy-4-ethyl-β-carboline-e-carboxylate), in contrast to benzodiazepines, was a potent convulsant *in vivo*. DMCM seemed to favor binding on a closed conformational state of the $GABA_A$ channel (Braestrup *et al.*, 1982). Shortly after, Costa and Hertz were pioneers in demonstrating that some, but not all δ-opioid receptor antagonists, had a "negative" intrinsic activity in membranes prepared from NG108-15, i.e. they reduce basal adenylyl cyclase activity (Costa and Hertz, 1989). Therefore, they proposed that the receptor could be under two states, only one in association with G-proteins, in the absence of the ligand. In a pharmacological model, Wreggett and De Lean predicted that "antagonists" may be active in hindering the ability of receptors to spontaneously associate with G-proteins (Wreggett and De Lean, 1984).

The existence of "inverse" agonists was a seminal observation that was pivotal for the existence of isomerization between R and R* (Chidiac *et al.*, 1994; de Ligt *et al.*, 2000).

However, it was not until mutated GPCRs were discovered, which were able to adopt active conformations in the absence of ligands and it was demonstrated that over-expression of native wild-type receptors generates enough active receptors to elicit a response, that the concept was fully accepted.

The first description of a mutated constitutively active receptor was made by Lefkowitz and collaborators (Cotecchia *et al.,* 1990; Kjelsberg *et al.*, 1992). They reported that mutations of a key residue (A^{293}) in the C-III loop, a region critically involved in the activation of G-proteins, are spontaneously active. The substitution of A^{293} for each of the other 19 amino-acid residues led to receptors having a variable, but always observable constitutive activity. This may indicate that in critical GPCRs domains, evolution has selected the residue which allows constraint upon the receptor under the R form (Samama *et al.*, 1993).

In certain human diseases, mutations of GPCRs are often the causal agents as we will discuss in section VIII (Spiegel, 1996).

In theory, mutated receptors have a less constrained form and therefore a higher potency to isomerize from R to R*. This is what we found by measuring the isomerization J constant in COS-cells for wild-type and constitutively active mutated 5-HT_4 receptors (Claeysen *et al.*, 2000). However, Claeysen et al. have also found that mutated receptors have different R* structures (Claeysen *et al.,* 2001).

The existence of an equilibrium between an inactive form R and an active form R*, governed by the equilibrium constant J, also explains that over-expression of wild-type GPCRs, by the virtue of increasing the absolute amount of R* (keeping the J constant unchanged), generates measurable constitutive activity, both in cell lines, but also *in vivo* (Chidiac *et al.*, 1994; Lefkowitz *et al.,* 1993). Bond et al. described transgenic mice over-expressing the wild β2−AR at various receptor levels in atrial cells (Bond *et al.,* 1995). Baseline, left atrial tension in these transgenic mice was increased 3-fold over control mice and ICI-118,551 acts as an

inverse agonist, decreasing basal tension. The degree of ICI-118,551 inhibition was correlated with receptor density, suggesting that the event was receptor-mediated.

In fact, some receptors are more or less constrained under the R form. In COS-7 cells, at a density of 300 fmol/mg (a density close to that found in wild-type tissues), the β2-AR had no constitutive activity, whereas the 5-HT_{4a}R increased cAMP production by 4-fold. Further increasing the density of β2-AR generates constitutive activity (Claeysen *et al.,* 1999). In contrast, the wild-type PACAP receptor will not generate a constitutive activity event at high density (unpublished results from our laboratory).

Interestingly, splice variants of GPCRs and especially those showing splicing within the C-terminal domain, have variable constitutive activities. There is increasing evidence that specific sequences of the C-terminal region of GPCRs modulate the isomerization between R and R* (Claeysen *et al.*, 1999; Joly *et al.*, 1995; Prézeau *et al.*, 1996). A cluster of basic residues present in the C-terminal of the short C-terminal forms of mGluR splice variants (mGluR1b, mGluR1c and mGluR1d) impair their ability to isomerize from R to R* (Mary *et al.*, 1998). Removing this cluster by mutation reveals their constitutive activity. In addition, the effect of this inhibitory cluster is suppressed by the long C-terminal domain of the other splice variant mGluR1a which has, in fact, a high constitutive activity (Mary *et al.*, 1998).

Considering the splice variants of 5-HT_4 receptors differing in the length of their C-terminal domain; the shorter the tail, the higher the constitutive activity. Artificial truncations revealed a domain of the tail, rich in Ser and Thr, which is largely engaged in constraining the receptor under the R form (Claeysen *et al.*, 1999).

Truncating or splicing out the last residues of thyrotropin-releasing hormone receptors and prostaglandin E receptors induces constitutive activity. The last 12 residues of bovine rhodopsin have also been proposed to be involved as negative regulators of the GTP

exchange. Removing the C-terminus of avian β-AR increases its activity (Hasegawa *et al.*, 1996; Matus-Leibovitch *et al.*, 1995; Parker and Ross, 1991; Weiss *et al.*, 1994).

Either C-terminal-associated proteins, or post-translational modifications in the C-terminal domain, are likely to be involved in the turning-off of constitutive GPCR activity. This remains to be demonstrated. However, the existence of such interacting proteins, able to reduce the isomerization from R to R*, is possible. Their presence in tissues, in which these receptors are naturally expressed, and not in cell lines, would also explain why it is easy to obtain constitutive activity of GPCRs transfected in the latter. Recently, we found that the Homer proteins which interact with the C-terminus of mGluR1,5 (see section XI and figure 7) are able to modulate the constitutive activity of these receptors (Ango *et al.*, 2001).

C. Constitutively active wild-type receptors in native tissues

Demonstrating the existence of constitutively active wild-type receptors in native tissues has in fact proved to be difficult (not speaking here of cell lines, naturally expressing some GPCRs). There are several reasons for this difficulty. The first is that due to the reasons discussed above, constitutive activity is low and undetectable in wild-type tissues. The second reason is that to demonstrate such an activity, one has to be sure that there are no agonists to contaminate the preparation. Finally, constitutive activity can only be demonstrated in native tissues if inverse agonists are available, which is only the case for some receptors. However, the list of GPCRs for which an inverse agonist is available is getting longer.

Inverse agonist effects have been observed in different cell lines without any need for over-expression of GPCRs (H.E.L. 92.1.7 cells endogenously expressing the human α2-AR, RIN5AH cells expressing α2-D receptors and, as already discussed, NG108-15 cells expressing δ-opioid GPCR) (see de Ligt *et al.*, 2000). In native tissues, there is convincing evidence that in myocytes, forskolin-stimulated Ca^{2+} currents can be inhibited by the β-

adrenergic atenolol in the absence of agonists. Similarly, atropine seems to be able to block basal Ca^{2+} channel activity. Bradykinin antagonists also seem to be able to decrease basal phospholipase C activity in rat myometrial cells expressing endogenous receptors (see (de Ligt *et al.*, 2000) for references). The overall demonstration of the existence of constitutively active GPCRs in native tissues is weak. However, Morisset et al. recently found that native histamine H3 receptors, which inhibit histamine release, are constitutively active. Physiologically, histamine H3 inverse agonists stimulated histamine release (Morisset *et al.*, 2000).

The idea that naturally existing "inverse agonists" can be found in some organisms, is becoming more and more popular (see de Ligt *et al.*, 2000). The agouti protein has been proposed to be an inverse agonist of melanocortin GPCRs and exendin, a peptide isolated from the lizard, has been proposed to be an inverse agonist of glucagon-like peptide GPCRs. We have already discussed the existence of constitutively active GPCR encoded within the genome of viruses such as Kaposi's sarcoma-associated herpes virus, poxvirus and cytomegalovirus. The herpes virus GPCR shows homology with vertebrate C-X-C receptors and is also referred to as ORF-74. An interferon-γ-inducible protein (IP10), agonist on C-X-C receptors, acts as an inverse agonist on ORF-74 (Geras-Raaka *et al.*, 1998). Other chemokines, such as growth-related oncogenes are agonists, whereas stromal cell-derived Factor1a (SDF1a) and viral macrophage inflammatory protein II (vMIP-II) are also inverse agonists of ORF-74 (Rosenkilde *et al.*, 1999). vMIP-II is also an antagonist of human chemokine GPCRs (Kledal *et al.*, 1997).

D. Effects of long-term treatment with inverse agonists

The possibility of categorizing GPCR antagonists into two categories: neutral antagonists and inverse agonists, is not without consequences. Indeed, there is data showing that chronic

treatment with antagonists and inverse agonists do not have similar long-term consequences. The classical effect of long-term treatment with inverse agonists is an up-regulation of receptors which could be more pronounced than the up-regulation observed with antagonists (MacEwan *et al.*, 1996; MacEwan and Milligan, 1996a; MacEwan and Milligan, 1996b). In both cases, stabilization of the occupied receptor occurs. Interestingly, long-term treatment with the 5-HT_{2C} inverse agonist, SB206553, sensitized the PLC pathway both to 5-HT, but also to ATP (Berg *et al.*, 1999). An up-regulation of Gαq/11 partially accounts for these observations. Similarly, Bouaboula et al. (Bouaboula *et al.*, 1999) also observed that treatment with a cannabinoid receptor (CB2) inverse agonist (SR 144528) also inhibits the lyso-phosphatidic acid (LPA) receptor-mediated pathway, an effect attributed to the trapping of Gαi on CB2 occupied by SR 144528 (Bouaboula *et al.*, 1999).

VIII. Diseases caused by GPCR mutations

In view of the physiological importance of GPCRs, it is not so surprising that a series of pathologies have been found to be related to mutations of GPCRs (Spiegel, 1996). Most of these pathologies are related to obvious clinical manifestations such as blindness, *diabetes insipidus*, hypo- or hyper-thyroidism, etc. Some mutations, as yet undiscovered, providing non-obvious phenotypes, are also likely to be responsible for pathologies such as psychiatric or neurological disorders. Some germline mutations may never be detected because they are incompatible with life. Both germline (inherited) and somatic (post-zygotic) mutations have been found to be responsible for loss or gain of functions.

A. Loss of function mutations

These mutations are generally inherited disorders. Among them, the most common ones are certainly those that affect the gene coding for rhodopsin and vasopressin V2 receptors which are responsible for retinitis pigmentosa (RP) (Dryja and Li, 1995) and nephrogenic diabetes insipidus (NDI), respectively (Arthus *et al.*, 2000) (see the web site: www.medcon.mcgill.ca/nephros). Approximately 100 and 117 mutant alleles have been identified as being causal agents in RP (autosomal dominant except for three recessive alleles) and NDI (X-linked and autosomal recessive) pathologies, respectively. In both cases several types of receptor abnormalities have been found.

A minority of RP exhibit no difference from wild-type receptors when studied in heterologous cells (Edwards *et al.,* 2000). In contrast, in photoreceptor cells, some of them (for example those affecting the last five residues of the C-terminal - truncation or point mutation), appear to have a mislocalization in the photoreceptor cell (Edwards *et al.*, 2000). They remain in the cell body, rather than in the membrane sacs within the rods (Dryja and Li, 1995). Indeed, as we will discuss in section XI, the C-terminus of rhodopsin interacts with TcTex-1, a dynein light chain subunit (Tai *et al.*, 1999) allowing the transport of post-Golgi rhodopsin-containing vesicles along the microtubules towards the outer segment. The majority of mutations (most are missense mutations) lead to non-functional rhodopsin acting as dominant negatives. Indeed, haplo-insufficiency is unlikely to be the problem since carriers of at least one apparent *null* allele are phenotypically normal (Rosenfeld *et al.*, 1992). In most cases, the protein is certainly misfolded and retained in the endoplasmic reticulum, causing photoreceptor degeneration.

In the majority of NDI mutants, the problem is the abnormal intracellular retention of the synthesized protein. The receptor is most probably misfolded. Interestingly, permeant but not impermeant V2 vasopressin receptor antagonists have been found to dramatically increase

cell-surface expression and rescue function of eight mutant vasopressin receptors, thus acting as pharmacological chaperones (Morello *et al.,* 2000). This opens new therapeutic avenues for NDI and also for other GPCR-related pathologies due to misfolding of the protein. Another therapeutic possibility has been proposed following the observation that co-transfection of mutated receptors (nonsense, frame-shift, deletion or missense mutations in the third intracellular loop or the last two TMs) with a C-terminus V2-R peptide spanning the sequence where the various mutations occur, rescues functional receptors (Schoneberg *et al.,* 1997; Schoneberg *et al.,* 1996).

The diseases caused by loss of function of mutant GPCRs also include:

- color blindness (cone opsins) (Nathans, 1999);
- familial ACTH resistance (ACTH-R) (Clark *et al.,* 1993; Naville *et al.,* 1996; Tsigos *et al.,* 1993);
- male pseudo-hermaphrodites under-virilization, or female amenorrhea with low estrogen production (LH-R) (Misrahi *et al.,* 1998; Themmen *et al.,* 1997);
- male defect in spermatogenesis and female insensitivity to FSH with ovarian dysfunction and infertility (FSH-R) (Misrahi *et al.*, 1998; Themmen *et al.*, 1997);
- familial or sporadic hypothyroidism (TSH-R) with phenotypes ranging from resistance to TSH to true hypothyroidism (Duprez *et al.,* 1999);
- familial hypocalciuric hypercalcaemia/neonatal severe primary hyper-parathyroidism (CASR) (Brown, 1999);
- congenital bleeding (Thromboxane A2-R) (Hirata *et al.,* 1994);
- Hirschprung's disease (endothelin-R) (Puffenberger *et al.,* 1994);
- Laron-type dwarfism (GH-R) (Godowski *et al.,* 1989; Okimura and Norton, 1998; Sanchez *et al.,* 1998; Wojcik *et al.,* 1998);

- familial GH deficiency (GHRH-R) (Maheshwari *et al.*, 1998; Salvatori *et al.*, 1999; Wajnrajch *et al.*, 1996);
- hypo-gonadism (GNRH-R), (Caron *et al.*, 1999; de Roux *et al.*, 1999a; de Roux *et al.*, 1999b);
- Blomstrand's chondrodysplasia (PTH/PTHr-R) (Zhang *et al.*, 1998);
- non-insulin-dependent diabetes mellitus (Glucagon-R) (Hager *et al.*, 1995);
- obesity (melanocortin 4-R) (Hinney *et al.*, 1999; Vaisse *et al.*, 1998; Yeo *et al.*, 1998); narcolepsy in Doberman dogs (orexin-R) (Lin *et al.*, 1999).

B. Gain of function mutations

Site-directed mutagenesis of adrenergic receptors of the C-III loop by Lefkowitz's group leads to the key notion that a mutated receptor can adopt an active R* conformation in the absence of the ligand (Cotecchia *et al.*, 1992; Kjelsberg *et al.*, 1992). Constitutively active mutated receptors responsible for diseases were described very rapidly after this discovery. One of them was the TSH receptor for which a wide series of somatic mutations have been described in many patients suffering from autonomous thyroid adenomas (Duprez *et al.*, 1999; Parma *et al.*, 1993; Parma *et al.*, 1995). The first described mutations were localized within the C-III loop and subsequent data indicate that a cluster of mutations is localized at the amino-terminal portion of TM-VI. However, other mutations are dispersed along the receptor in: the extracellular domain, the extracellular loops, and TM-II, III, V, VI and VII (Duprez *et al.*, 1999). Hereditary, as well as sporadic toxic thyroid hyperplasia with growing goiter and absence of auto-immunity, characterizing Graves disease, have been associated with mutations of TSH-R (Duprez *et al.*, 1999). Familial gestational hyperthyroidism can be observed in the absence of auto-immunity and normal levels of hCG. A mutation affecting the extracellular domain of the TSH-R has been described. This mutation displaying an increased

sensitivity to stimulation by hCG, keeps the same response to TSH (Rodien *et al.,* 1998). The second receptor for which a constitutive activation has been described is the LH-R which causes familial male precocious puberty (Shenker *et al.,* 1993; Themmen *et al.,* 1997). Interestingly, there are no clinical manifestations in female carriers of the mutated gene. Spontaneous LH-R activation is sufficient to stimulate testosterone production and spermatogenesis, but inefficient in triggering puberty in females without concomitant FSH function.

The diseases caused by gain of functions of mutant GPCRs also include:

- congenital night blindness (rhodopsin) (Robinson *et al.,* 1992). In the Lys^{296}-Met mutant in rhodopsin cannot bind 11-cis-retinal, the receptor is constitutively active and this leads to retinal degeneration (Robinson *et al.,* 1992). This is supposed to be equivalent to constant light exposure which is known to cause photoreceptor cell death;
- hypoparathyroidism (CASR) (Brown and Hebert, 1997);
- Jansen metaphyseal chondrodysplasia (PTH/PTHrP) (Schipani *et al.,* 1995; Schipani *et al.,* 1996);
- pigmentation (somber and tobacco darkening) defect in mice (MSH-R) (Robbins *et al.,* 1993);

Genetic polymorphism of GPCRs frequently occurs (in contrast to rarer occurring mutations) and most probably underlies inter-individual variability in both pharmacological responses and propensity to diseases:

- a polymorphism in β2-adrenergic-R (Arg^{16}-Gly) occurs more frequently in patients with nocturnal asthma, but its occurrence in hyper-tensive subjects is controversial (Buscher *et al.,* 1999; Strosberg, 1997);
- a polymorphism (Trp^{64}-Arg) of β3-AR may be associated with earlier onset of non-insulin-dependent diabetes and morbid obesity;

- the dopamine D4-R contains an unusual polymorphism constituted of a 16-residue repeat region in the C-III loop. There are some pharmacological differences between the 4- and the 7-repeat receptors. A group of subjects with the 7 repeat allele exhibit significantly elevated Novelty Seeking scores in comparison to subjects lacking the 7 repeat allele (Ebstein *et al.*, 1996). However, no link has been found between the 7 repeat allele and the deficit / hyperactivity disorder (Swanson *et al.*, 2000).

IX. Allosteric antagonists and agonists of GPCRs

We will call allosteric ligands those which do not bind to the binding site of the natural ligands.

A long series of non-peptidic antagonists for peptide GPCRs have been discovered, thanks to high throughput screening. They generally bind to the central core, composed of the 7 TMs. Non-peptidic antagonists for angiotensin (AT1 and AT2), bombesin, bradykinin, endothelin, opiates, vasopressin, oxytocin, neurotensin, substance P and CRF, are now available (Betancur *et al.*, 1997). As opposed to peptidic antagonists, they have the advantage of crossing the blood brain barrier. In fact, one may recall that for opiate GPCRs, allosteric agonists (morphine and heroin) and allosteric antagonists (naloxone) have been known for a long time. Non-peptidic antagonists do not have the same binding site as peptidic agonists. For example, CP96345 inhibits the NK1 substance P receptor with a Ki of 14 nM. However, mutations localized in the extracellular regions of TM-V and TM-VI, suppress CP96345 binding without modifying the binding of substance P (Gether *et al.*, 1993). The structural modifications of these regions are indeed crucial for receptor activation. Introducing histidines into these regions, followed by Zn^{++} treatment, inhibits receptor activation, but not substance P binding (Elling *et al.*, 1995). Steroid hormones may also possibly bind to the core domain of GPCRs acting as allosteric antagonists. This has been shown in the case of

oxytocin-R which is blocked by progesterone (Grazzini *et al.,* 1998). Aspirin and sodium salicylate allosterically inhibit endothelin receptors (Talbodec *et al.,* 2000).

Interestingly, small and possibly allosteric antagonists of chemokine receptors, like bicyclam derivatives for CXCR4 or UCB35625 for CXCR3, are likely to be future compounds used to block HIV infection (De Clercq, 2000; Sabroe *et al.,* 2000). Allosteric compounds acting on muscarinic receptors have also been described (see Lazareno *et al.,* 2000).

In the case of family 3, high affinity competitive antagonists have been particularly difficult to find. This is probably due to the difficulty of modifying the structure of natural ligands, while maintaining their affinity. Recently, novel, subtype-selective group I mGluR non-competitive antagonists have been discovered which bind within the core 7TM domain (which has not been involved in binding glutamate so far). CPCCOEt and BAY 36-7620 are specific mGluR1 non-competitive antagonists, whereas MPEP is a mGluR5 non-competitive antagonist (Carroll *et al.,* 2001; Litschig *et al.,* 1998; Pagano *et al.,* 2000). Synthetic molecules like NPS467 and NPS568 have been shown to potentiate the action of Ca^{2+} on CASR (Hammerland *et al.,* 1999; Nemeth *et al.,* 1998). Alone, neither compound has agonist effects, but both induce a shift to the left of the Ca^{2+} concentration response curves, indicating that they act as allosteric modulators. Ca^{2+} alone may be a positive allosteric ligand acting at a site close to the binding site of glutamate in mGluRs (Kubo *et al.,* 1998), and GABA in $GABA_B$ receptors (Galvez *et al.,* 2000b).

X. Post-trancriptional and post-translational modifications of GPCRs: role in activation and desensitization

We have seen in section V that GPCR - G-protein interactions are complex and diverse. In addition to splicing with insertions localized mainly at the C-III loop and the C-terminal

domains, GPCR domains implicated in G-protein interactions are susceptible to fine tuning via post-transcriptional and post-translational modifications.

A. RNA editing

Transcripts encoding the 5-HT_{2C} receptor, a PLC- and phospholipase A2-coupled receptor, undergo RNA editing events in which the genomically-encoded adenosine residues are converted to inosines by double-stranded RNA adenosine deaminase(s). Seven major 5-HT_{2C}-R isoforms are predicted, encoded by 11 distinct RNA species, differing in their second intracellular loops. These modifications lead to changes in the efficacy of coupling to G-proteins. Agonist stimulation of the non-edited human receptor (5-HT_{2C}-INI) and the edited 5-HT_{2C}-VSV and 5-HT_{2C}-VGV receptor variants stably expressed in NIH-3T3 fibroblasts, demonstrate that serotoninergic agonists were less potent at the edited receptors (Burns *et al.*, 1997). This reduced G-protein coupling for the edited isoforms is primarily due to silencing of the constitutive activity of the non-edited 5-HT_{2C}-R.

B. Phosphorylation associated with desensitization

Several mechanisms regulate the length and strength of GPCR signals. For a series of recent reviews see (Bohm *et al.*, 1997; Bunemann and Hosey, 1999; Bunemann *et al.*, 1999; Pitcher *et al.*, 1998; Tsao and Zastrow, 2000). Generally, desensitization refers to a progressive loss of the physiological response and the effector activity, despite the continued presence of the signaling ligand. Two types of desensitization have been described. Homologous desensitization refers to the situation whereby only the activated GPCRs desensitize, while heterologous desensitization refers to the situation whereby activation of one GPCR leads to the desensitization of responses initiated by another, heterologous GPCR. There are multiple mechanisms of receptor desensitization, but they are not all understood. The most rapid phase

of desensitization (minutes) involves agonist-induced phosphorylation either by classical second messenger activated kinases (mainly in heterologous desensitization) or by GPCR-activated kinases (GRK), followed by binding to adapter proteins such as arrestins. Sequestration or internalization is a second event that commonly occurs with a slightly slower time-course. The receptor can be recycled at the cell surface, or destroyed in lysosomes (down-regulation).

In heterologous desensitization, classical second messenger-activated protein kinases are implicated. β2-ARs are excellent substrates for cAMP protein kinase (PKA) and their phosphorylation, on the C-III loop, leads to an uncoupling from Gs. Recent reports suggest that PKA-mediated phosphorylation can increase β2-adrenergic receptor coupling to Gi and promote MAP-kinase cascade activation (Daaka *et al.*, 1997; Luttrell *et al.*, 1999). However, this may not be a general mechanism (Luttrell *et al.*, 1999; Tsao and Zastrow, 2000). PKC-mediated heterologous desensitization has also been reported (Bunemann *et al.*, 1999). A complex between the A-kinase-anchoring protein, AKAP79/150, PKA and PKC, facilitates receptor phosphorylation.

Homologous desensitization appears to involve phosphorylation by a unique family of GRKs (Pitcher *et al.*, 1998). Six GRKs have been identified: GRK1 (rhodopsin-kinase localized in the retina), GRK2, 3 (β-adrenergic receptor-kinases, β-ARK1, 2), GRK4, 5, 6. GRK4 is localized in testis, the others have a wide distribution.

The mechanisms of association of GRKs to the plasma membrane are diverse. GRK1 is isoprenylated, whereas GRK4 and 6 are palmitoylated. GRK2 and 3 are translocated to the membrane when βγ is released from G-proteins upon activation by the phospholipid environment. Phosphorylation by GRKs occurs either in the C-terminus domain [(β2-AR, rhodopsin, somatostatin (SSTR3)), or in the C-III loop (α2-AR, muscarinic-R (m2)]. GRKs

are subject to regulation by PKA, PKC and GRK themselves and Ca^{2+} binding proteins such as recoverin (for GRK1), calmodulin or others (Pitcher *et al.,* 1998).

Phosphorylation does not necessarily mean uncoupling. After GRK-induced phosphorylation of rhodopsin and β2-AR, uncoupling occurs when arrestins (4 arrestins have been identified, arrestin-1 being found only in the retina) bind to the phosphorylated receptors. Other uncoupling or adapter proteins may exist. The most classical pathway for β2-AR endocytosis involves its targeting to coated pits, although it can also be targeted, as some other GPCRs, to caveolae (Tsao and Zastrow, 2000). The targeting to coated pits is associated with the binding of several proteins to arrestin, such as the AP-2 adapter, NSF (N-ethylmaleimide-sensitive fusion protein), clathrin and Src (Laporte *et al.,* 2000; Laporte *et al.,* 1999; McDonald *et al.,* 1999; Tsao and Zastrow, 2000). For some, but not all muscarinic receptors, dynamin, a GTPase that forms the necks of the vesicles, is required both in coated pits and caveolae-mediated endocytosis (see for example Ahn *et al.,* 1999; Bunemann *et al.,* 1999). Internalization of Src via GPCR-induced endocytosis has been proposed to be the mechanism by which these receptors activate the MAP-kinase cascade. This hypothesis is questioned by other studies.

After being internalized in endosomes, the receptors can be recycled following dephosphorylation by a specific member of phosphatase 2A and 2B families. Recycling of β2-AR requires interaction of its PDZ C-terminus ligand binding domain with the EBP_{50}/NEHRF erzin-actin complex (see section X) (Cao *et al.,* 1999). Alternatively, the receptor (vasopressin V2) can be sequestered for a long time in an unknown intracellular compartment (Innamorati *et al.,* 1999; Oakley *et al.,* 1999), or degraded in lysosomes (down-regulation) or in non-lysosomal compartments (Tsao and Zastrow, 2000). Whether the pathways involved in endocytosis and down-regulation are always similar or not, remains to be clarified. Down-regulation of β2-AR also involves a decrease in receptor synthesis and destabilization of

mRNA (Bohm *et al.*, 1997). Divergent residues located at the C-terminus of thrombin and substance P receptors specify differences in trafficking between lysosomal and recycling pathways.

The role of GRKs has also been studied *in vivo* using transgenic mice. GRK2 knock-out mice were found to be lethal to the embryo at the age of 15 days. They had severe defects in cardiac development and most likely died of cardiac failure. Mice over-expressing GRK2 showed an increased cardiac function (Pitcher *et al.*, 1998). GRK3 (β-arrestin2) determines morphine tolerance, but not dependence (Bohn *et al.*, 2000).

XI. GPCRs are unfaithful to G-proteins

It will come as no surprise to the reader to realize that GPCRs interact with many other proteins, in addition to G-proteins (Hall *et al.*, 1999). This has already been discussed in the section on desensitization, where GPCRs have also been shown to interact with arrestins. This is a consequence of receptor activation via G-proteins and will not be discussed further here. What is more surprising is that GPCRs also directly interact with a wide variety of proteins which are important for some of their functions (clustering, anchoring, optimization of transduction, transduction not mediated through G-proteins…) and which do not implicate G-proteins.

A. Association with PDZ domain-containing proteins

Various proteins have been recognized to contain PDZ domains; 90-residue domains first recognized in PSD-95/SAP90, its Drosophila homologue. Three groups of PDZ ligands have been recognized: Group I PDZ binds to C-terminal peptides with the consensus E-(S/T)-X-Φ; in most cases, Φ is V or I) group II binds peptides with the Φ-x-Φ triplet and group III binds

peptides with a less defined consensus sequence such as ϕϕΦ, as exemplified in β-neurexine YYV (Φ being a hydrophobic residue and ϕ being an aromatic residue) (Daniels *et al.,* 1998; Maximov *et al.,* 1999; Sudol, 1998a). However, some variations on this theme are likely. Many ionic channels, receptor channels, enzymes, and synaptic protein C-termini, contain a PDZ binding sequence which interacts with the PDZ of multi-domain proteins. They form vast complexes in the synapse (Ehlers *et al.,* 1998; O'Brien *et al.,* 1998).

Many GPCR C-termini also contain PDZ binding ligand (figures 6 and 7):

- β2-AR associates via a C-terminal PDZ binding domain with the Na^+/H^+ exchanger regulator factor (NEHRF) and its homologue, the erzin-radixin-moesin binding phosphoprotein-50 (EBP-50) (figure 6A) (Cao *et al.*, 1999; Hall *et al.*, 1998a; Hall *et al.*, 1998b). This protein contains two PDZ, the first one interacts with the DSLL C-terminus sequence of β2-AR. The interaction of β2-AR and NEHRF has solved a mystery in the β2-AR signaling pathway involved in the regulation of the Na^+/H^+ transporter. Indeed, for a long time, β2-AR were thought to be only coupled to Gs and that the only second messenger implied was cAMP. However, deletion of parts of the C-III intracellular loop of β2-AR suppressed the Gs coupling as expected, but kept the activation of the Na^+/H^+ exchange by β2-AR agonists (Barber *et al.*, 1992). This was a clear indication as to the existence of another signaling pathway not requiring Gs and the C-III loop. Hall et al. using a β2-AR tail-GST protein fished out NEHRF (Hall *et al.*, 1998a). In the absence of β2-AR agonists, NEHRF binds to the Na^+/H^+ exchanger and inhibits its activity (figure 6A). NEHRF associated to activated β2-AR, suppresses the inhibition of NEHRF, leading to stimulation of the Na^+/H^+ exchanger (figure 6A). Mutation experiments indicate that the PDZ binding domain of β2-AR is involved. P2Y1-R and the cystic fibrosis TM regulator also interact via their PDZ binding domains with the NEHRF protein (Hall *et al.*, 1998b). In addition to the regulation of the Na^+/H^+ exchange, the β2-AR- EBP-50 erzin-actin complex controls the

recycling of the receptor after endocytosis. Phosphorylation of the serine (Ser^{411}) of the DSLL binding domain by GRK-5, inhibits the association of β2-AR with EBP_{50}/NEHRF and receptor recycling (Cao *et al.,* 1999).

- Somatostatin acts through five different GPCRs (SSTR1-R5). The SSTR2 has been shown to interact via its QTLI -PDZ binding domain, with two related proteins, cortactin-binding protein 1 (CortBP1) and SSTRIP (figure 6B) (Zitzer *et al.,* 1999a; Zitzer *et al.,* 1999b). In fact, CortBP1 and SSTRIP multi-domain proteins originate from the same family. CortBP1 is also called Shank2 and is homologous to ProSAP1, whereas SSTRIP is homologous to Shank1, Spank1, as well as synamon (see figure 7). These proteins contain various domains such as an ankyrine domain at the N-terminus, an SH3 domain, a PDZ domain, a proline-rich domain to which cortactin binds and a sterile a-motif (SAM domain) at its C-terminus which is implicated in dimerization (figure 7). SSTR2 associated to CortBP1 is stimulated by somatostatin. SSTR2 associates with actin via cortactin, an interaction proposed to be implicated in its anchoring in nerve terminals, where it regulates neurotransmitter release via the inhibition of N-terminal calcium channels.
- Using the two-hybrid screen, in which the C-terminus of the $5\text{-}HT_{2C}$ receptor was a bait, MUPP1 (multi-PDZ-domain protein), a 13-PDZ domain protein was isolated (Ullmer *et al.,* 1998). Protein alignment of all PDZ domains from INADL (inactivating no after-potential D-like), C52A11.4 and MUPP1, revealed that all three proteins share extremely identical PDZ domains. Moreover, the most identical PDZ domains are arrayed in the same order resulting in the same organization. $5\text{-}HT_{2C, 2B, 2A}$ receptors interact with PDZ 10 (figure 6C) via their C-terminus SSV, SYV and SCV, respectively. The MUPP1-$5\text{-}HT_{2C}$ interaction was demonstrated in transfected COS-cells, and also in choroid plexus. Co-clustering and change in the conformation of MUPP1 occurs during the interaction (Bécamel *et al.,* 2001) MUPP1 also interacts with NG2 via PDZ1 and with c-Kit via its PDZ 10 (Barritt *et al.,* 2000;

Mancini *et al.,* 2000). The discovery of additional proteins that interact with other PDZ domains of MUPP1 should reveal its real function.

Note that re-sensitization and coupling of 5-HT_2-R to NO-S have been reported to implicate the C-terminus PDZ ligand of 5-HT_2-R. For these 5-HT_2-R-mediated transduction processes, the interacting PDZ protein has been identified (Backstrom *et al.,* 2000; Manivet *et al.,* 2000).

- The N-terminus PDZ domain of RGS12 has been shown to interact with a PDZ binding sequence of the A/S-T-X-(L/V) type domain. The search for the possible interacting proteins indicates that the C-terminus of the interleukine-8 receptor B (CXCR2) and that of the alternative 3' exon form of RGS12, are likely to be partners (Snow *et al.,* 1998).
- mGluR7a receptors interact with the protein interacting C kinase (PICK1), a one PDZ binding protein, via their C-terminus (-LVI). A much larger C-terminal domain is needed for a tight binding. The PICK1 PDZ site also interacts with AMPA receptors, ephrin ligands and receptors, and class I ADP-ribosylation factors. Since PICK1 can dimerize, a clustering of these molecules is likely. PICK1 interacts with the C-terminal of PKCα (QSAV) and modulates the phosphorylation of mGluR7a by PKCα (Dev *et al.,* 2000).
- The Drosophila photo-transduction cascade is not a cGMP phosphodiesterase-mediated pathway, but a PLC-β pathway leading activation of store-operated Ca^{2+} entry channels (TRP) and their associated non-specific cationic conductance channel TRPL (light activated channels) (Montell, 1997). PLC-β is activated by the rhodopsin-Gq complex (Tsunoda *et al.,* 1997; Xu *et al.,* 1998). A remarkable characteristic of this cascade is the fact that rhodopsin, Gαq, PLC-β, TRP, TRPL and PKC all bind (directly or indirectly) with INAD (inactivating no after-potential D), a 5 PDZ domain containing protein) (Bahner *et al.,* 2000; Montell, 1997). TRP and TRPL clearly interact via their C-terminal PDZ binding domains, however, the mode of interaction of other proteins in the cascade is not as obvious. If one notes that INAD forms homo-multimers, then a vast complex is clearly involved (transduciosomes).

INAD also interacts with calmodulin and an unconventional myosin: NINAC (Montell, 1997).

B. Association with EVH1-domain containing proteins

A family of proteins which appear to function as a link between cell surface signals (including focal adhesion proteins, zyxin and vinculin, or the axon guidance receptors, SAX-3/Robo) and actin-based cytoskeleton has recently been described. These proteins include Drosophila-enabled (Ena termed Mena in mouse), yeast Bee1p, vasodilator-stimulated phospho-protein (VASP) and the Wiscott-Aldrich syndrome protein (WASp) and encode one EVH1 domain which binds the consensus proline-rich motif FPPPP (Prehoda *et al.,* 1999).

The Homer protein family belongs to the VASP/WASp super-family, but contains another type of EVH1 domain with a slightly different structure. This domain that binds the consensus sequence (PPXXF) has already been described (Beneken *et al.,* 2000). The first member, called Homer1a, Vesl-1S and Ania-3 (named respectively by the three groups involved in its discovery), was identified on the basis of the rapid up-regulation of its mRNA (immediate early gene) following seizures, long-term potentiation, cocaine- and dopaminergic D1-stimulation (Berke *et al.,* 1998; Brakeman *et al.,* 1997; Kato *et al.,* 1997). Homer1a can be considered as an immediate early gene (IEG). The N-terminus (110 residues) essentially contains the EVH1 domain which has been shown to interact with group I mGluRs, IP3 and ryanodine receptors as well as Shank (CortBP1/ProSAP1/spank/SSTRIP) proteins (figure 7) (Naisbitt *et al.,* 1999; Tu *et al.,* 1999; Tu *et al.,* 1998; Xiao *et al.,* 1998). The other members of the Homer family contain an additional C-terminus with a predicted coiled-coil (CC) structure, including leucine zippers which allow them to dimerize or multimerize. These members comprise: 1) two other Homer1 splice variants, differing by 12 residues [Homer1b, and Homer1c (= PSD-Zip45, = Vesl-1L)]; 2) two Homer2 splice variants, differing by 11

residues [Homer2a (= cupidin, = Vesl-2D11 and Homer2b (= Vesl-2)]; 3) one Homer3. One Drosophila Homer (D-Homer) has also been cloned, as well as other splice variants (Fagni *et al.*, 2000; Kato *et al.,* 1998; Shiraishi *et al.,* 1999; Tadokoro *et al.*, 1999; Xiao *et al.*, 1998; Xiao *et al.,* 2000).

The PPSPF domain of mGluR5, approximately 50 residues from the carboxyl terminus (figure 7) is essential for binding the EVH1 domain of Homers, whereas its C-terminus PDZ ligand may interact with the PDZ domain of Shank (Naisbitt *et al.*, 1999; Xiao *et al.*, 2000). If one also notes that NMDA receptors also interact with this Shank PDZ domain via the PSD-95 - GKAP (guanylate kinase associated protein) heterodimer, and that Shank dimerizes and also interacts with Cortactin and therefore actin (figure 7), this constitutes a colossal post-synaptic complex. The Homer dimers, or multimers, may directly connect mGluR group I to IP3 and ryanodine receptors, shown to be engaged in their signaling pathways. Induction of Homer1a, which does not dimerize (because it does not contain the CC domain), following active neuronal activation, has been shown to modify the mGluR1-induced kinetic of intracellular Ca^{2+} release (Tu *et al.*, 1998). A role of Homers in cellular trafficking has also been reported (Xiao *et al.*, 2000). In cerebellar neurons, Homer1b and Homer1a are required to address a stabilized mGluR5 to dendrites, as well as dendrites plus axons, respectively (Ango *et al.,* 2000).

C. Association with enzymes and transcription factors

Several GPCRs, and especially angiotensin AT1 and 5-HT_{2A}, have been shown to activate the Jak kinases, better known as the first element in the cytokine receptor signaling cascade. These two receptors have been shown to bind Jak kinase in an agonist-dependent manner (Guillet-Deniau *et al.,* 1997; Marrero *et al.,* 1995). In AT1 receptors, the critical binding motif (YIPP) is localized within the proximal part of the C-terminus (319-322) (Ali *et al.,* 1997).

Tyrosine phosphorylation of this motif by Src, is necessary for binding the SH2 domain of SHP-2 (tyrosine phosphatase), which then binds Jak/STAT. The same phosphorylated tyrosine is necessary for the binding of PLC-γ1 on the same YIPP motif (Venema *et al.*, 1998). An interaction of CXCR4 receptors and Jak2/Jak3 kinases has also been reported (Vila-Coro *et al.*, 1999).

Another interesting case is the association of bradykinin B2 receptor with eNOS and nNOS (endothelium and neuronal nitric oxide synthases) (Golser *et al.*, 2000; Ju *et al.*, 1998). eNOS forms an inhibitory complex with bradykinin receptors, likely via the blockade of the flavin to heme electron transfer. The inhibitory complex is released in a ligand- and Ca^{2+}-dependent manner, due to tyrosine phosphorylation of the eNOS interacting region of the receptor. A similar interaction of angiotensin AT1 and endothelin-1 ETB receptors with eNOS has been described (Marrero *et al.*, 1999). In all three receptors, the receptor-interacting domain is localized within the C-IV region (Ju *et al.*, 1998).

Interestingly, the C-termini of $GABA_BR1$ and R2, which contribute to their hetero-dimerization, (as seen in section IV), are engaged in an interaction with the leucine-zipper domain of ATF-4 (CREB2), a transcription factor of the CREB/ATF family. ATF-4 and $GABA_B$ receptors are co-localized within soma and dendrites of cultured hippocampal neurons. Nehring et al., as well as White et al., demonstrated that $GABA_B$ receptors activate ATF-4-translocation to the nucleus and gene transcription in heterologous systems (Nehring *et al.*, 2000; White *et al.*, 2000).

D. Association with arrestins and associated proteins

We have already discussed that arrestins bind to phosphorylated GPCRs. This event is the first step in endocytosis (see section X). Arrestins recruit several other proteins, such as c-Src, NSF, AP2 and clathrin. This complex and the Src induced phosphorylating dynamin, within

the coated pits, lead to the formation of endocytotic vesicles. Endocytosis of Src, leading to the activation of the MAP kinase cascade mediated via GPCR, has been proposed (see section XII and figure 8).

E. Miscellaneous

Interestingly, the C-IV region is also a binding site for Ca^{2+}/calmodulin in mGluR7 (O'Connor *et al.,* 1999). This binding appears to be competitive with Gβγ and is blocked following PKC phosphorylation of serine residues within the same region (Nakajima *et al.,* 1999). O'Connor et al. proposed a mechanism by which Ca^{2+}/calmodulin is required to release Gβγ from the C-terminus of mGluR7 in order to obtain a Gβγ-mediated N-type Ca^{2+} channel (O'Connor *et al.,* 1999). Similarly, two regions of mGluR5 (and mGluR1a but not mGluR1b,c) interact with Ca^{2+}/calmodulin (Minakami *et al.,* 1997). These regions are localized within the C-terminus (842-869 and 922-950) in mGluR5.

A domain, 10 residues upstream from the last C-terminal domain (339-348) of the angiotensin AT1 receptor, interacts with another protein, ATRAP (AT1 receptor-associated protein) (Daviet *et al.,* 1999). Functional studies suggest that this protein inhibits AT1 coupling to PLC.

The C-terminus of rhodopsin (Gln^{344}-VAPA) is highly conserved among vertebrates and is a hot spot for mutations that cause some forms of *retinitis pigmentosa* (RP). Two of these C-termini mutants (Gln^{344}-ter characterized by removing the last 5 residues and Pro^{347}-Leu), are characterized by a specific defect in the transport of rhodopsin (Sung *et al.,* 1994; Tai *et al.,* 1999). Mice that express Gln^{344}-ter rhodopsin show abnormal rhodopsin accumulation in the plasma membrane (instead of outer segment discs) and cell body where it is synthesized (Sung *et al.,* 1994). This shows that the C-terminus is required for efficient transportation to, or retention in, the outer segment. Recently, the C-terminus has been shown to interact with

TcTex-1, a dynein light chain subunit (Tai *et al.*, 1999). This allows the transport of post-Golgi rhodopsin containing vesicles along the microtubules up to the outer segment.

β2-AR can be endocytosed following insulin or IGF-1 phosphorylation on Tyr^{350}. This creates a SH2 binding site for Grb2 which, via its SH3 domains, recruits PI3-kinase and dynamin. Induced β2-AR internalization by insulin requires the presence of this Tyr^{350} (Karoor *et al.*, 1998).

We can also recall that the C-terminus of dopamine D5, but not D1 receptors interacts directly with the γ2 subunit of $GABA_A$ receptors (Liu *et al.*, 2000).

F. Proteins interacting with the C-III loop

As we have seen, the C-III intracellular loop is very important for coupling to G-proteins. Proteins have been shown to interact directly to C-III of GPCRs. Spinophilin interacts with C-III of D2-dopaminergic receptors (Smith *et al.*, 1999), the ξ isoform of 14-3-3 proteins with α2-AR (Prézeau *et al.*, 1999) and endophilins with β1-AR. Spinophilin is a protein containing an N-terminus actin binding domain, a central PDZ domain and a coiled-coil C-terminus domain. The C-III of D2-dopaminergic receptors interacts with the domain of spinophilin localized between the actin binding domain and the PDZ domain which also binds protein phosphatase-1. It is interesting to note that two isoforms of D2 receptors are generated by alternative splicing. They differ by the presence (D2L) or absence (D2S) of 29 amino acids. It has recently been shown that D2L acts mainly at post-synaptic sites (Usiello *et al.*, 2000).

Endophilins 1/2/3, an SH3 domain-containing family, interact with a C-III polyproline domain of β1-AR not present in β2-AR. This interaction may be involved in internalization and desensitization of β1-AR. The roles of these proteins remain to be elucidated.

G. Proteins interacting with TM domains

There is much evidence for regulation of small G-proteins by heterotrimeric G-proteins, such as Ras, Rab, Rho, and ARF, but this generally occurs via a signaling cascade localized downstream of GPCRs. However, Rho and ARF have been shown to be immuno-precipitated in an agonist-dependent manner in association with m3 muscarinic receptors and AT1 angiotensin receptors. The common receptor sequence (NPxxY) required for the small G-protein interaction is localized at the end of the TM-VII and is also required to activate phospholipase D via these receptors. Direct interaction is likely but remains to be demonstrated (Mitchell *et al.*, 1998).

We have already discussed the interactions of the TM domains with other GPCRs in an homo-meric or heterotrimeric manner as well as with one TM protein (see section IV).

XII. Cross-talk between the GPCRs and the Tyrosine receptor kinase (TRK) and small G-proteins

In association with TRKs or alone, GPCRs and heterotrimeric G-proteins have recently been recognized to control proliferation, differentiation and even transformation (Gudermann *et al.*, 2000) (figure 8).

Two main signaling pathways are involved in these functions, the Ras and Rho pathways. TRKs are classically involved in activating the Ras pathway. The cascade includes auto-tyrosine phosphorylation of the receptors, tyrosine phosphorylation and association with proteins like Shc (SH2 domain-containing α2-collagen-related) and Grb2 (growth factor-bound protein 2), and recruitment of exchange factors for Ras-like SOS. Ras-GTP engaged the ERK (extracellular signal-regulated kinase) subfamily of MAPKs (mitogen-activated protein kinases). This includes the association with Raf1 or B-Raf kinases. Raf kinases are

serine/threonine kinases which phosphorylate MEK (ERK kinase), which subsequently phosphorylate ERKs. Translocation of ERKs into the nucleus controls the transcription of genes involved in division or differentiation. The kinetics of ERK activation, the concomitant activation of Rap1, another small G-protein and the absence or the presence of B-Raf, determine whether a TRK will induce proliferation (like EGF and PDGF) or differentiation (like NGF) (Gudermann *et al.*, 2000).

GPCRs acting via the four main subfamilies of heterotrimeric G-proteins, Gs, Gi/Go, Gq/G11 and G12/G13 control the TRK-ERK kinase pathway.

A. Gs coupled receptors

We have already discussed the fact that cAMP in some cells induces cell proliferation, whereas in many others cAMP is inhibitory. In cells like thyroid epithelium and GH secreting cells, gain of function of Gs lead to adenoma formation. In fact, cAMP via PKA may either inhibit Raf1 and therefore ERK and cell proliferation, like in NIH-3T3 fibroblasts (Chen and Iyengar, 1994), or activate ERK via activation of Rap1 and B-Raf (Vossler *et al.,* 1997). In neuronal cells B-Raf is the most important MEK activator and cAMP always activates ERK. cAMP seems to activate Rap1 without requiring the activation of PKA. Indeed, a cAMP Rap1 GEF (guanine nucleotide exchange factor) which is directly activated by cAMP, also named Epac (exchange protein directly activated by cAMP), has been described (de Rooij *et al.,* 1998; Kawasaki *et al.,* 1998). β2-AR activates ERK via Rap1 and B-Raf in HEK cells (Schmitt and Stork, 2000).

B. Gi/Go coupled receptors

The descriptions of tumors induced by constitutively active Gαi are very limited and are described only in human ovarian sex cord stromal tumors and adrenal cortical tumors (for a

review see (Gudermann *et al.*, 2000). However, many GPCRs like LPA receptors, α2-AR and 5-HT_{1A} receptors have been shown to activate the ERK pathway and cell division very efficiently via PTX-dependent Gi/Go (Gudermann *et al.*, 2000; Varrault *et al.*, 1992). In addition, the β2-AR Gs-coupled GPCR has also been shown to activate the ERK pathway via Gi/Go after its agonist induced phosphorylation (Daaka *et al.*, 1997). Gβγ is the active dimer after receptor activation. Several possible pathways are conceivable. We have already discussed the fact that Gβγ binds GRK leading to receptor phosphorylation, binding of arrestin and recruitment of Src and clathrin (see section XI). The endocytosis of the complex, via coated-pits, conveys Src in the cytoplasm. Src phosphorylates Sch which is a link to the Ras-ERK cascade (Luttrell *et al.*, 1998). However, some Gi/Go coupled GPCRs, like GnRH, activate ERK probably without requiring arrestin. Using dominant negative dynamin mutants which block endocytosis, some, but not all authors report an inhibition of GPCR or EGF-mediated ERK activation (see Tsao and Zastrow, 2000). Therefore, the requirement for endocytosis may not be a generalized mechanism of ERK activation by GPCRs. Gβγ which also activates PI3-kinase γ, may directly activate MEK via the PKC ζ (Takeda *et al.*, 1999). GPCRs, like angiotensin AT1, LPA and thrombin receptors, can also transphosphorylate RTKs via Src and unknown kinases (Heeneman *et al.*, 2000; Zwick *et al.*, 1999). Gαo/i may also activate the Rap1 pathway by sequestering Rap-GAP (Mochizuki *et al.*, 1999). Gβγ also activates Cdc42 (Simon *et al.*, 1995) and binds Rho and Rac (Harhammer *et al.*, 1996) and Arf (ADP ribosylation factor) (Franco *et al.*, 1995), involved in coat formation and vesicular trafficking.

C. Gq/ G11 coupled receptors

There are many pathways by which Gq/G11 activating GPCRs can activate the ERK pathway (Gudermann *et al.*, 2000). RasCalDAG-GEFI, II, III Ras GEF (GEF = guanine nucleotide

exchange factor also called GRF = GDP releasing factor), predominantly expressed in the nervous system are activated by DAG and Ca^{2+} (Yamashita *et al.,* 2000), whereas Ras GRF is a Ras GEF activated by Ca^{2+}-calmodulin in neuronal cells (Mattingly and Macara, 1996). In addition, the CalDAG-GEFI, III are also Rap1 GEF (Yamashita *et al.*, 2000). More directly, PKC has been shown to directly activate Raf-1 and the PYK2-Src interaction (Dikic *et al.,* 1996).

D. G12/G13 coupled receptors

Human G12 has been clearly identified as an oncogenic protein in NIH-3T3 cells (Chan *et al.,* 1993). GTPase-deficient Gα12 and Gα13 have been reported by several laboratories to be very efficient in transforming proteins. Gα12 has been identified as a transforming oncogene in Ewing's sarcoma, and the term gep oncogene has been attributed to Gα12 (Xu *et al.,* 1994). GPCR-mediated activation of G13 (and to a lesser extent G12) leads to p115RHoGEF, a RGS for these heterotrimeric G-proteins and a Rho GEF factor (Hart *et al.,* 1998; Kozasa *et al.,* 1998). Similarly, another G12/G13 RGS (PDZ-RhoGEF) is also a RhoGEF (Fukuhara *et al.,* 1999). GPCR-induced Rho activation may induce cytoskeletal rearrangements. However, the mechanisms by which this activation of Rho control growth proliferation remain unclear. In some cases, such as LPA, bradykinin (B2) and serotonin (5-HT_2) GPCRs, the involvement of EGF receptors is required to activate Rho. Rho regulates transcriptional events via serum-responsive element (SRE) and activates ROCK-I (Rho-associated coiled-coil forming kinase), two events which are likely to play a role in cell division (Gudermann *et al.*, 2000). In addition, G12 interacts directly with PLCε, a novel PLC member which is a RasGEF (Lopez *et al.,* 2001).

Concluding remarks

The GPCR saga started 25 years ago with a very simple question : how do hormones act at the cellular level? Thus, the chapter on cellular signaling was opened. Landmarks of this saga were the discovery of cAMP, the discovery and purification of heterotrimeric G-proteins, the purification and cloning of GPCRs and finally the crystal structure of rhodopsin. Cellular signaling and cell-cell communication is now the central question in biology which will remain until physiological functions and regulations, in particular brain functions ("the" cell-cell communication problem) and pathological diseases such as cancer, are fully understood.

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Legends to figures

Figure 1 - The "ménage à quatre" of GPCRs

A. GPCRs are transmembrane proteins (TM-I to TM-VII) with three extracellular loops (E-I to E-III) and three cytoplasmic loops (C-I to C-III), a fourth loop after TM-VII is present in rhodopsin (see figure 2D), and probably also in many family 1 GPCRs. GPCRs have been tinkered with by evolution to recognize ligands as different as photons, Ca^{2+}, amino acids, proteins, lipids… (Bockaert and Pin, 1999).

B. Activation cycle of heterotrimeric (αβγ) G-proteins by GPCRs. The GTPase activity of Gαs is activated by RGS (regulation of G-protein signaling). The different families of G-proteins and some of their effectors are listed.

Figure 2 - Structures of G-proteins and rhodopsin

A. Alignment of structural domains of the Gα subunit. The cylinders and the arrows represent the α-helix ad the β-strands, respectively. Linkers are illustrated in black. The domains of interaction with βγ and GPCRs are indicated. The ras domain and helical domain (green) are regionalised. SI, SII, and SIII are the "switch" domains which are structurally modified during the GDP → GTP exchange (see text).

B. Schematic representation of the structure of Gαs.

C. Complex between a GPCR, Gα and Gβ and Gβγ. GPCR is a ribbon illustration of rhodopsin. The 3D model of the receptor and heterotrimeric G-proteins has been generated using the coordinate of bovine rhodopsin and a Gtα/Giα chimera and βγ (code pdb1F88 and 1GOT, respectively) with Swiss Pdb viewer (Glaxo) and POV-Ray

D. Ribbon illustrations of rhodopsin, parallel to the plane of the membrane. Reprinted by permission from Science (Palczewski et al. 289, 739-745) copyright (2000) published by the American Association for the Advancement of Science.

E. A view into the membrane plane seen from the cytoplasmic side (extracellular side). Reprinted by permission from Science (Palczewski et al. 289, 739-745) copyright (2000) published by the American Association for the Advancement of Science.

Figure 3 - The different GPCRs

Protein sequences excluding N-terminal and C-terminal domains) were multi-aligned and a tree was calculated using ClustalW. A bootstrap analysis was performed on the tree construction; all branches with a bootstrap value lower than 80% were considered unreliable and were assigned a null length. The tree was drawn using TreeView (Page, 1996; Thompson *et al.*, 1997).

The accession numbers of GPCRs used to construct this tree are:

Family 1: 5-HT_4 P97288; adrenergic (β2) Q28044; adenosine (A1) P47745; rhodopsin P35362; EDG-1 (receptor for sphingosine-1 phosphate) M31210; TSH P16473; LH-HCG P22888.

Family 2: calcitonin-R P79222; CRF-R (corticotropin releasing factor 2) Q13324; secretin-R 47872; VIP-R (vasoactive intestinal peptide) P32241; glucagon-R P30082; EMR1 (cell surface glycoprotein F4/80) Q61549; α-latrotoxin-R O88927; BAI-1 (brain specific angio-genesis inhibitor) O14514; GPR56 AF106858.

Family 3: mGluR1 Q13255; VR4 (vertebrate putative pheromone receptor) O35192; TR1, TR2 (vertebrate putative taste receptor) Q9Z0R8; Boss-Drome (bride of sevenless protein of Drosophila melanogaster) P22815; Boss-Drovi (Boss protein of Drosophila virilis) Q24738; $GABA_B$-R1 O08620; CASR P35384.

Family 4: VN1-3 (putative pheromone receptors) Q62850, Q62856, Q62852.

Family 5: FZ-1 (vertebrate frizzled receptors) Q08463; FZD2, FZD3, 6 (Drosophila frizzled receptors) Q94916, O00144, O60353; MOM-5 (*Caenorhabditis elegans* frizzled-like receptor) O16147; LIN-17 (*Caenorhabditis elegans* frizzled-like receptor) U63557; SMO-Drome (Drosophila melanogaster smoothened receptor) P91682.

Family 6: CAR1,2,3,4 (cAMP receptor of *Dictyostelium discoideum*) P13773, P34907, P35352, Q9TX43.

Family 7: T2R1, 3, 4 (bitter taste receptor) AAF43902, AAF43903, AAF43904.

Family 8: DOR (Drosophila odorant receptor) P81909, P81910, P81914, P81915, P81921.

Extra family receptors:

Yeast pheromone receptors: STE2 P06842; STE3 P06783; Pheromone P Q00619.

Putative receptors from *Arabidopsis thaliana*: O04214

Mth: Methuselah putative GPCRs O97148.

Putative Drosophila gustative receptors: GR59D1 AC O86245; GR59D2 AC006245; GR21D1 AC004420; GR22B1 AC003945.

Figure 4 - Crystal structure of mGluR1 fly-trap domains in liganded (glutamate) states.

A. The two protomers (in violet, left and red on the right), are shown from a position parallel to the membrane. The LB1 is on the top and LB2 at the bottom of the figure. Glutamate is made up of spheres. The 3D model has been generated using the coordinates of the mGluR1 extracellular domain (code pdb1EWK) with Swiss Pdb Viewer (Glaxo).

B. Illustration of the spatial arrangements of the two mGluR1 protomers. The two parts of LB1 (LB1a and LB1b) and LB2 (LB2a and LB2b) domains as well as the linkers (1,2,3) and the cysteine-rich region (CR) are indicated.

Figure 5 - The central core of family 1 GPCRs

A. Inactive family 1 GPCRs (view from the cytoplasm). The TM included in the N- and C-terminal part of C-I and C-III which are considered to be α-helical structures (modified from Bourne, 1997b). The bars represent the structural constrains between the a-helical structures. The dotted line represents the structural constrains between TM-VII and TM-III provided by the retinal. For a further description of the other constrains, see section V.

B. Activation of the central core of family 1 GPCRs (view from the cytoplasm). During activation, some of the constrains are released. TM-VII rotates clock-wise (30°). A cysteine introduced at position 285 goes from a hydrophobic domain to the hydrophilic cleft. The distance between TM-III and TM-VI increases. This opens a cleft in the central core in which the G-proteins can find their way. For a further description, see section V.

Figure 6 - GPCR interactions with PDZ proteins.

For further information, see section XI.

Figure 7 - The glutamate post-synaptic synapse.

For a complete description, see section XI.

Figure 8 - GPCR-receptor tyrosine kinase (RTK) dialog for activation of extracellular receptor kinases (ERKs) and cell division or differentiation.

For a complete description, see section XII.

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Abbreviations

α-AR:	α-adrenergic receptor
AC:	Adenylyl cyclase
ACTH:	Adreno-corticotropin hormone
AGS:	Activator of G protein signaling
AID:	Acquired immuno-deficiency
AKAP:	A-kinase anchoring protein
AlF4-:	Fluoroaluminate
AP2:	adaptin 2
APC:	Adenomatous polyposis coli
ARF:	ADP-ribosylation factor
AT:	Angiotensin
ATP:	Adenosine trisphosphate
BAI-1:	Brain specific angio-genesis inhibitor
β-AR:	β-adrenergic receptor
β-ARK:	β-adrenergic receptor kinase
BRET:	Bioluminescence resonance energy transfer
CASR:	Calcium sensing receptor
CB2:	Cannabinoid receptor 2
CCR4:	Chemokine receptor 4
CortBP1:	Cortactin binding protein 1
CRF:	Corticotropin-releasing factor
CRLR:	Calcitonin receptor-like receptor
DAG:	Diacylglycerol

DMCM: Methyl 6,7-dimethoxy-4-ethyl-β-carboline-e-carboxylate

EBP: Erzin-radixin-moesin binding protein-50

EGF: Epidermal growth factor

EMR1: Human cell specific surface glycoprotein F4/80

Ena: Drosophila enabled

eNOS: Endothelial-type NO-synthase

Epac: Exchange protein directly activated by cAMP

ERK: Extracellular regulated kinase

EST: Expressed sequence tags

EVH1: Ena/WASp homology domain 1

FRET: Fluorescence resonance energy transfer

FSH: Follicle-stimulating hormone

GABA: Gamma aminobutyric acid

GAIP: Gα-interacting protein

GAP: GTPase activating protein

Gαt: Transducin

GEF: Guanine nucleotide exchange factor

GH: Growth hormone

GHRH: Growth hormone releasing hormone

GIRK: G-protein-coupled inward rectifying potassium channel

GKAP: Guanylate kinase-associated protein

GnRH: Gonadotropin releasing hormone

GPCR: G-protein coupled receptors

G protein: GTP binding protein

GR: Gustatory receptor

GRF:	GDP releasing factor
GRK:	GPCR-activated kinases
GDP:	Guanosine diphosphate
GTP:	Guanosine triphosphate
hCG:	Human chorionic gonadotrophin
HEK:	Human embryonic kidney (cells)
HIV:	Human immuno-deficiency virus
HPLC:	High performance liquid chromatography
IEG:	Immediate early gene
INAD:	Inactivating no after-potential
IP3:	Inositol trisphosphate
IP10:	Interferon-γ-inducible protein
LH:	Luteneizing hormone
LIN-7:	*Caenorhabditis elegans* frizzled-like receptor
LNB-TM-VII:	Seven TM domains containing a long N-terminal extracellular region
LPA:	Lyso-phosphatidic acid
LPS:	Lipopolysaccharide
mGluR:	Metabotropic glutamate receptor
MIP-1α	Macrophage inflammatory protein-1α
MOM-5:	*Caenorhabditis elegans* frizzled-like receptor
MUPP1:	Multi-PDZ domain protein
NDI:	Nephrogenic *diabetes insipidus*
NEHRF:	Na^+/H^+ exchanger regulator factor
NGF:	Nerve growth factor
NK1:	Neurokinin 1

nNOS: Neuronal-type NO synthase

NSF: N-ethylmaleimide-sensitive fusion protein

P2Y-h: Purinergic P2Y-receptor

PACAP: Pituitary adenylate cyclase activating polypeptide

PAR: Protease activated GPCRs

PDE: Phosphodiesterase

PDGF: Platelet-derived growth factor

PDZ: A domain present in **P**SD95 and its Drosophila analog **D**isc-large tumor suppressor gene and in tight junction protein **Z**O-1

PICK: Protein interacting C kinase

PCK: Protein kinase C

PLC: Phospholipase C

PM: Plasma membrane

ProSAP: Proline-rich synapse-associated protein

PrP: Prion protein

PTH: Parathyroid hormone

R: Receptor under an inactive state

R*: Receptor under an active state

RAMP: Receptor activity-modifying protein

RANTES: Regulated upon activation, normal T-cell expressed and secreted

RGS: Regulator of G protein signaling

ROCK: Rho-activated coiled-coil forming kinase

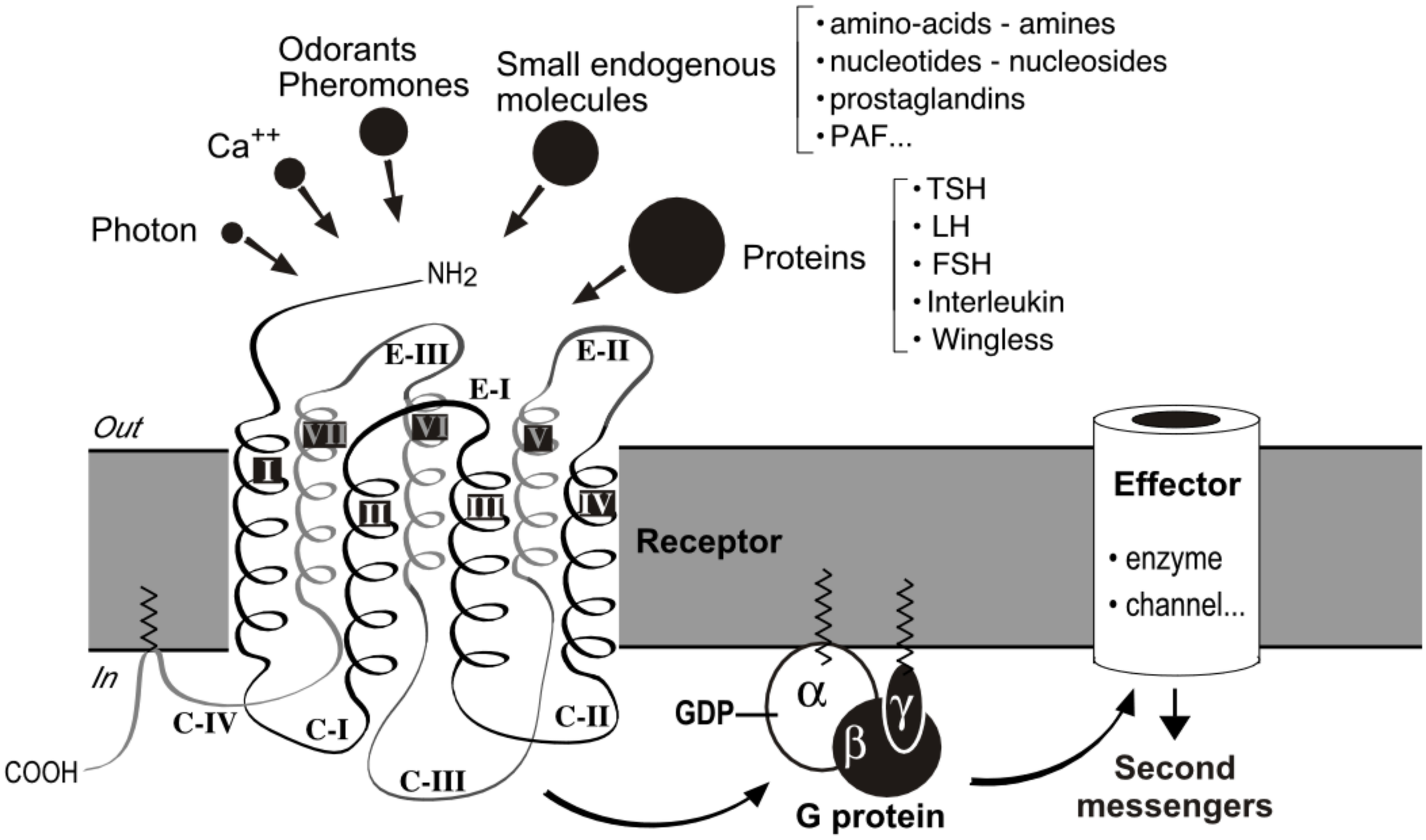
RP: *Retinitis pigmentosa*

SDF-1: Stromal derived factor-1

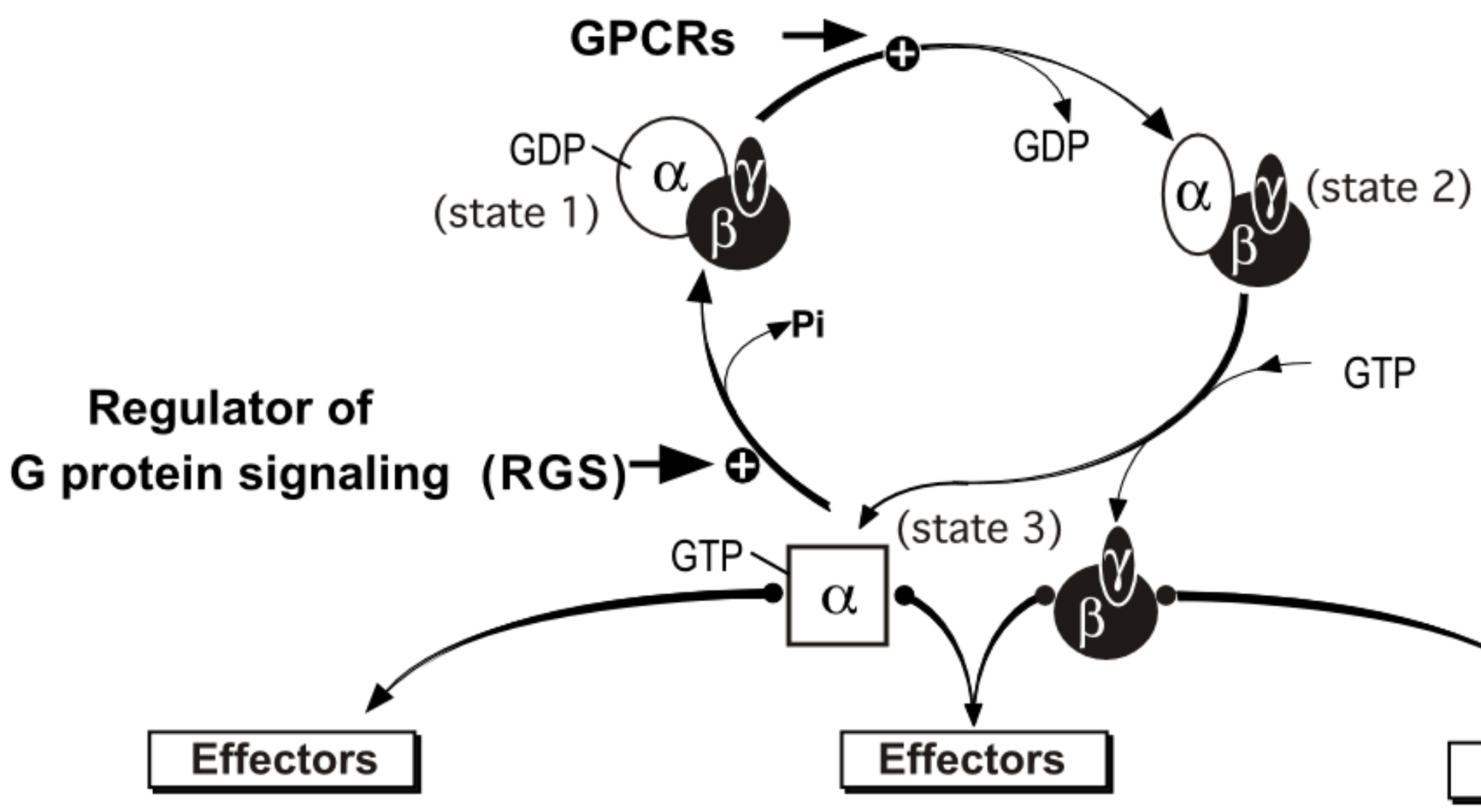
SH3: Scr homology domain 3

SST:	Somatostatin
SST2:	Somatostatin receptor 2
SSTRIP:	Somatostatin interacting protein
TM:	Transmembrane domain
TNF:	Tumor necrosis factor
TRK:	Tyrosine receptor kinase
TRP:	Transient receptor potential calcium channel
TSH:	Thyroid-stimulating hormone
VASP:	Vasodilator-stimulated phospho-protein
VIP:	Vasoactive intestinal peptide
WASp:	Wiskott-Aldrich syndrome protein
WD repeat:	Tryptophan-aspartate repeat

A



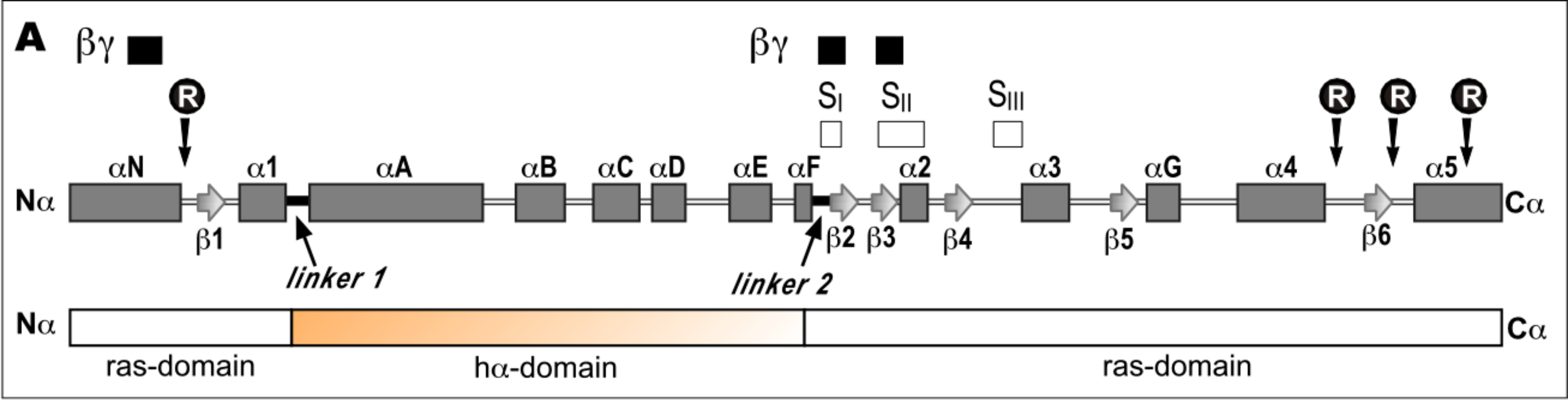
B

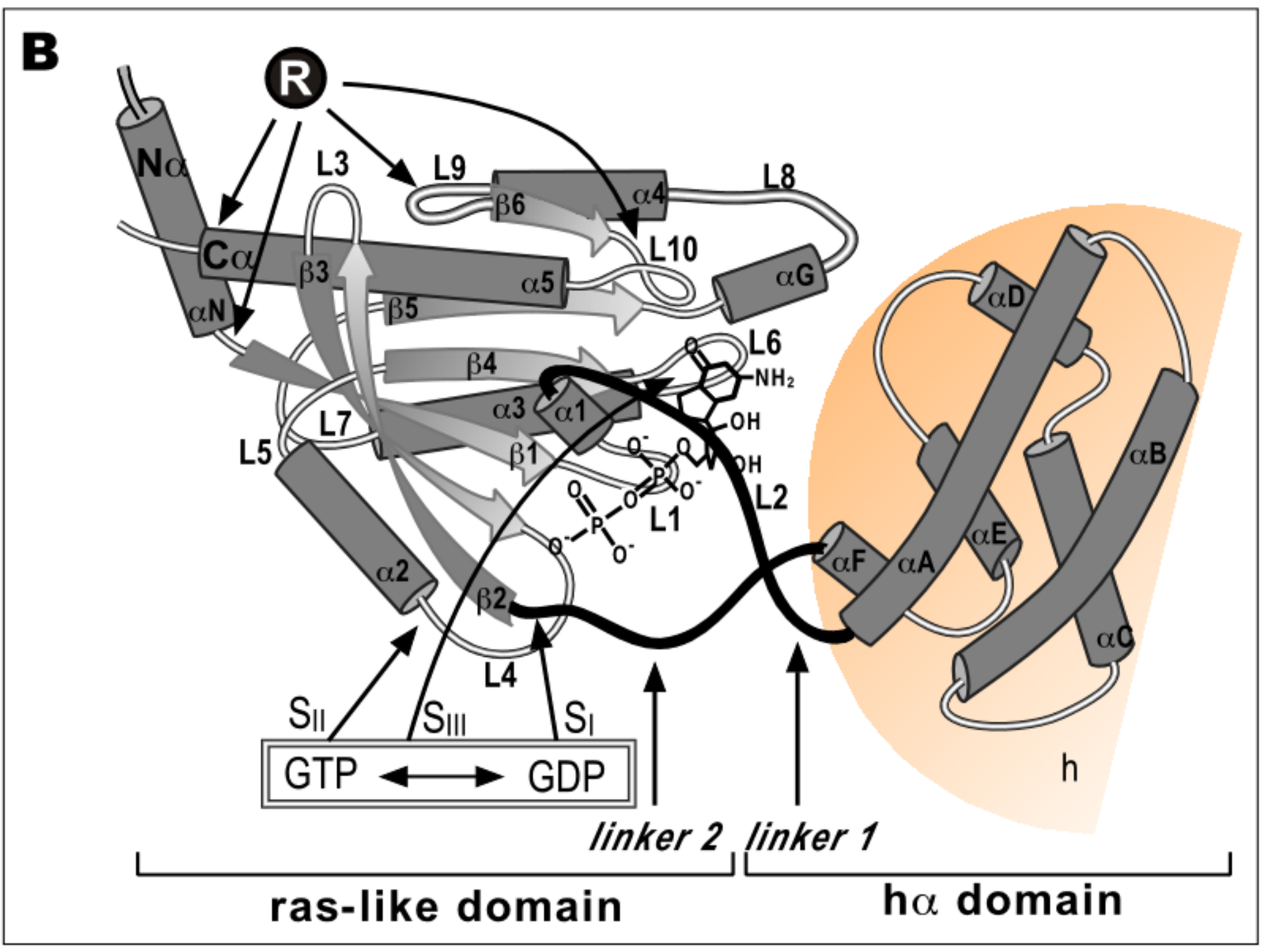


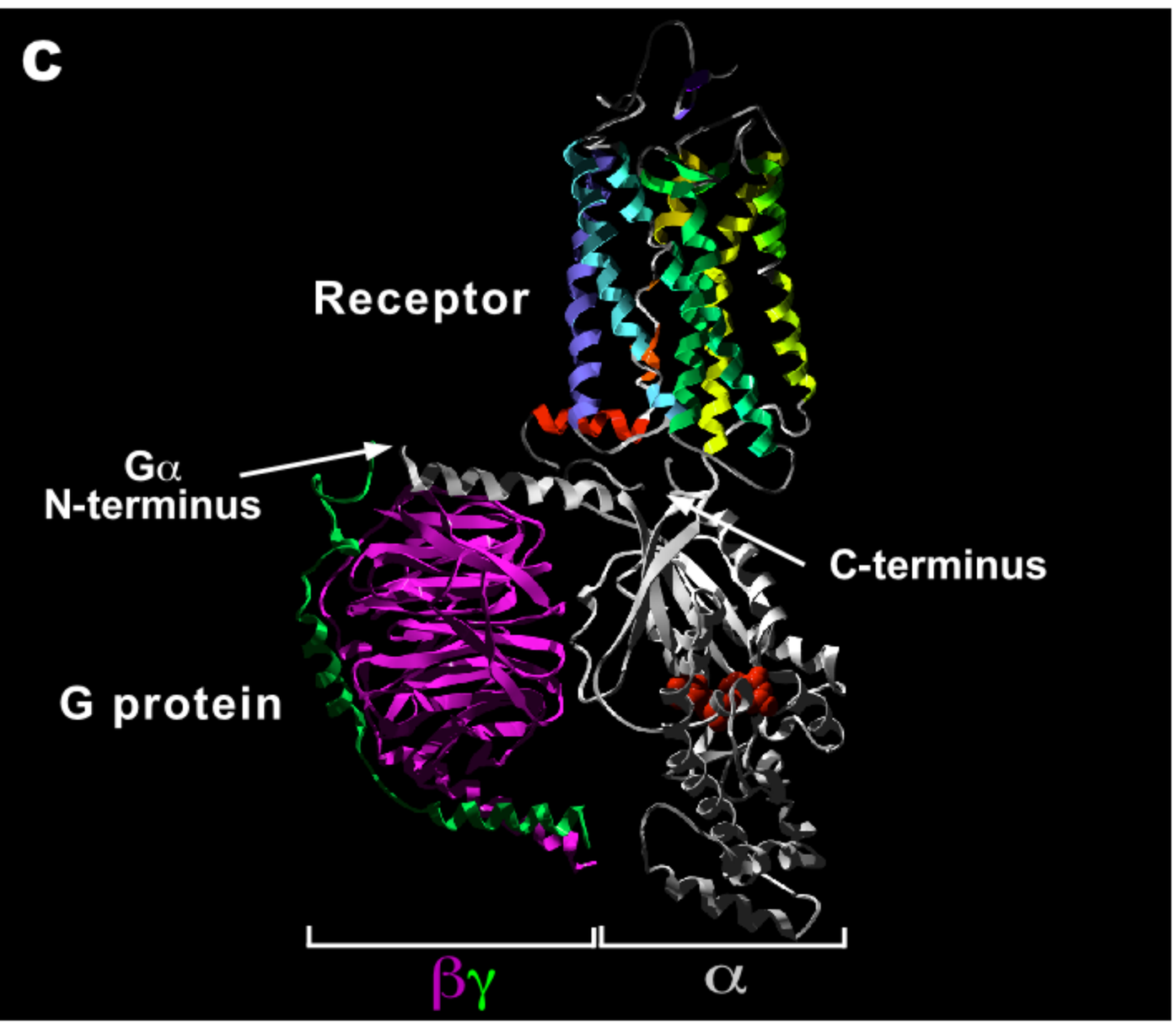
family					
α_s		Adenylyl-cyclases (all)	(+)	Adenylyl-cyclase (II, IV)	(+)
				L type Ca^{++} channels	(+)
α_i	α_t α-Gust	cGMP- phosphodiesterase	(+)		
	$\alpha_{i,\ 1,2,3}$	Adenylyl-cyclases (I, V, VI)	(-)		
	α_o	Adenylyl-cyclase (I)	(-)		
		Ca^{++} channels	(-)		
α_q	α_q, α_{11} $\alpha_{14\text{-}16}$	Phospholipases C (β_1 ,β_3)	(+)		
α_{12}	α_{12}, α_{13}	Phospholipase A_2	(+)		
		Rac-cdc 42	(+)		
		Na^+/ H^+ exchanger	(+)		
		Phospholipase C (ε)	(+)		
		Rho-GEFs	(+)		

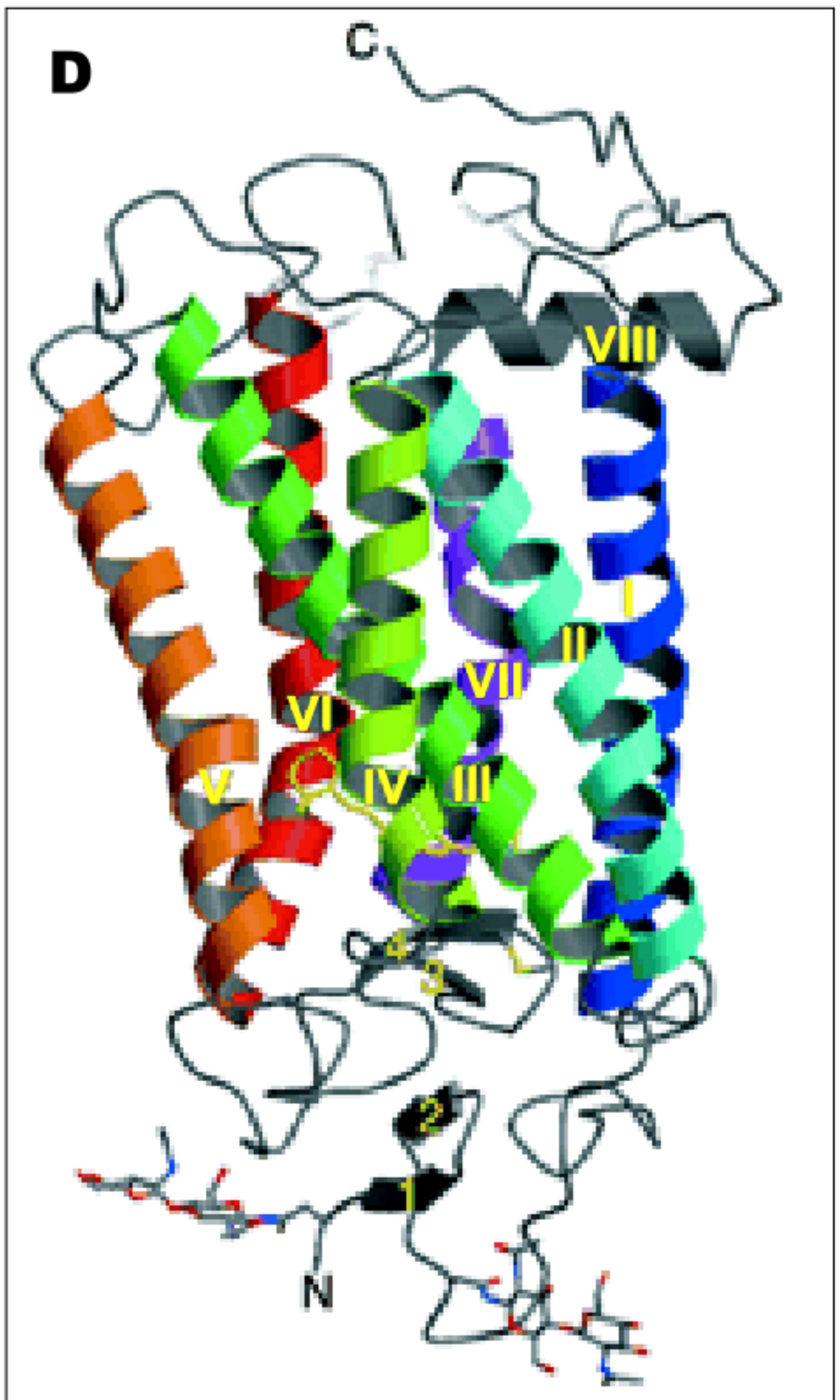
GIRK	(+)	
Phospholipase C (β_2)	(+)	
Ca^{++} channels (N/P)	(-)	
Adenylyl-cyclase (I)	(-)	
Phospholipase A_2	(+)	
β-ARKinases	(+)	
MAP-Kinases	(+)	
PI_3-Kinases	(+)	

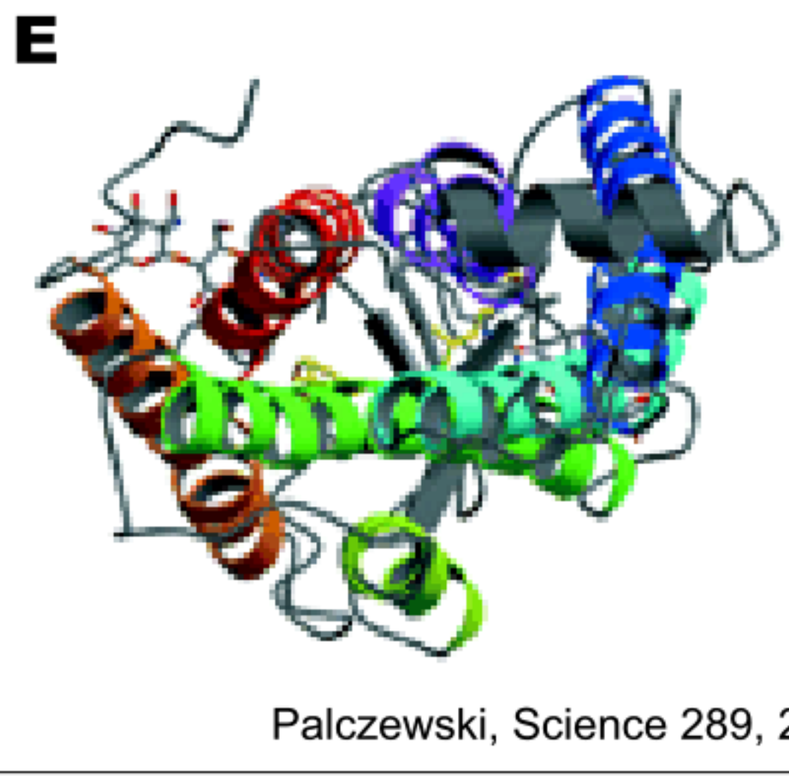
Figure 1











Palczewski, Science 289, 2000

Figure 2

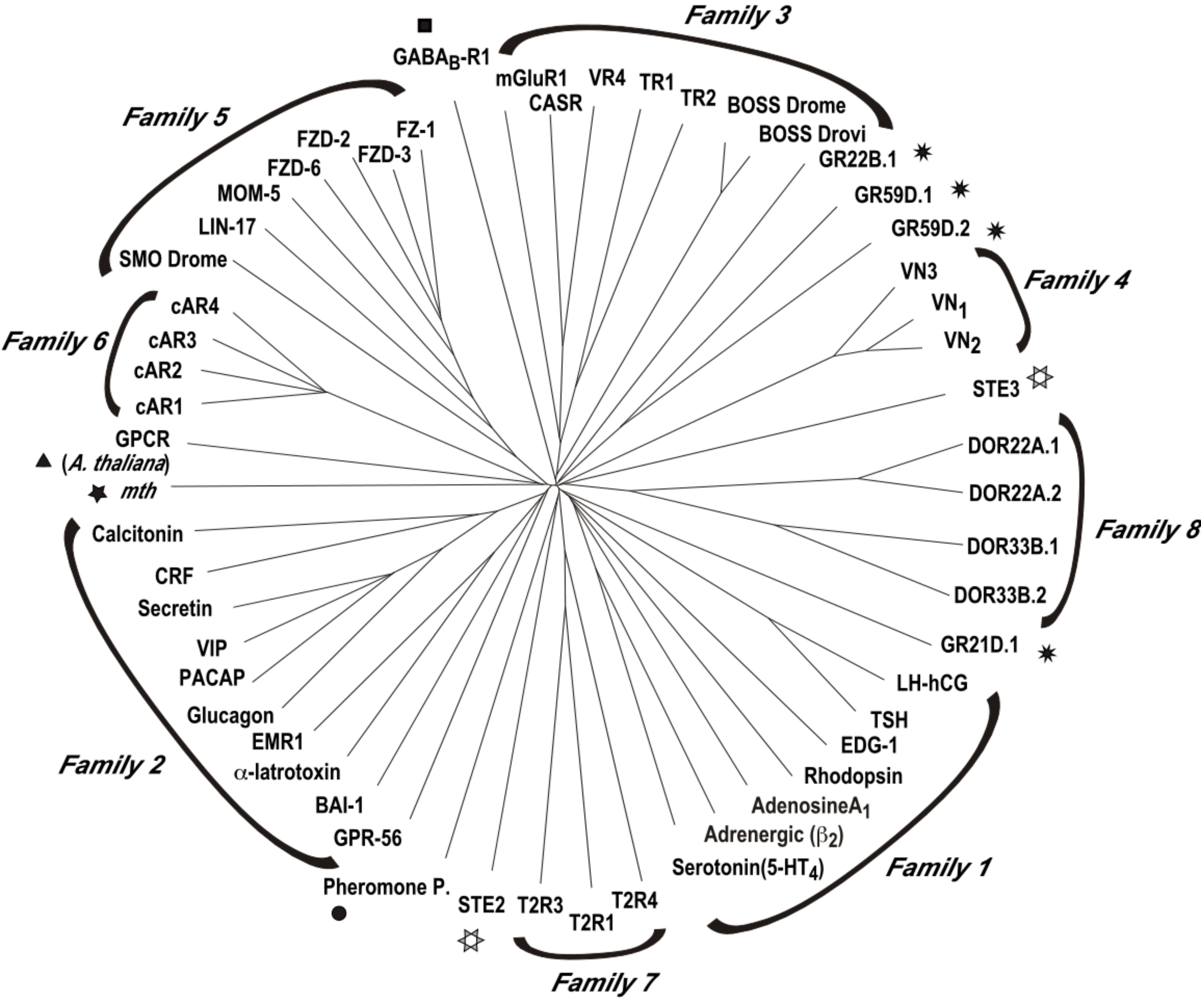

Family 3
GABA$_B$-R1
mGluR1
VR4
TR1
TR2
CASR
BOSS Drome
BOSS Drovi
GR22B.1
GR59D.1
GR59D.2
Family 4
VN3
VN$_1$
VN$_2$
STE3
Family 5
FZ-1
FZD-2
FZD-3
FZD-6
MOM-5
LIN-17
SMO Drome
Family 6
cAR4
cAR3
cAR2
cAR1
GPCR
(A. thaliana)
mth
DOR22A.1
DOR22A.2
DOR33B.1
DOR33B.2
GR21D.1
Family 8
Calcitonin
CRF
Secretin
VIP
PACAP
Glucagon
EMR1
α-latrotoxin
BAI-1
GPR-56
Family 2
LH-hCG
TSH
EDG-1
Rhodopsin
AdenosineA$_1$
Adrenergic (β$_2$)
Serotonin(5-HT$_4$)
Family 1
Pheromone P.
STE2
T2R3
T2R1
T2R4
Family 7

Figure 3

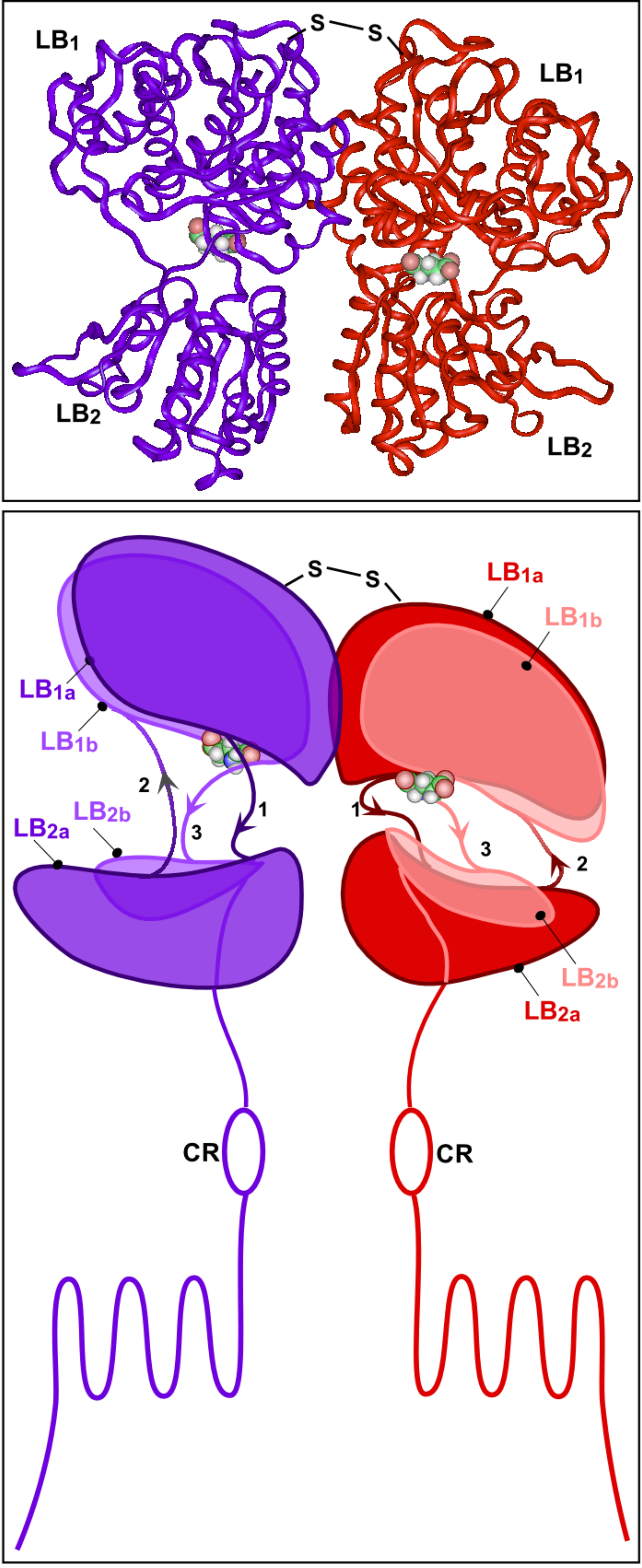
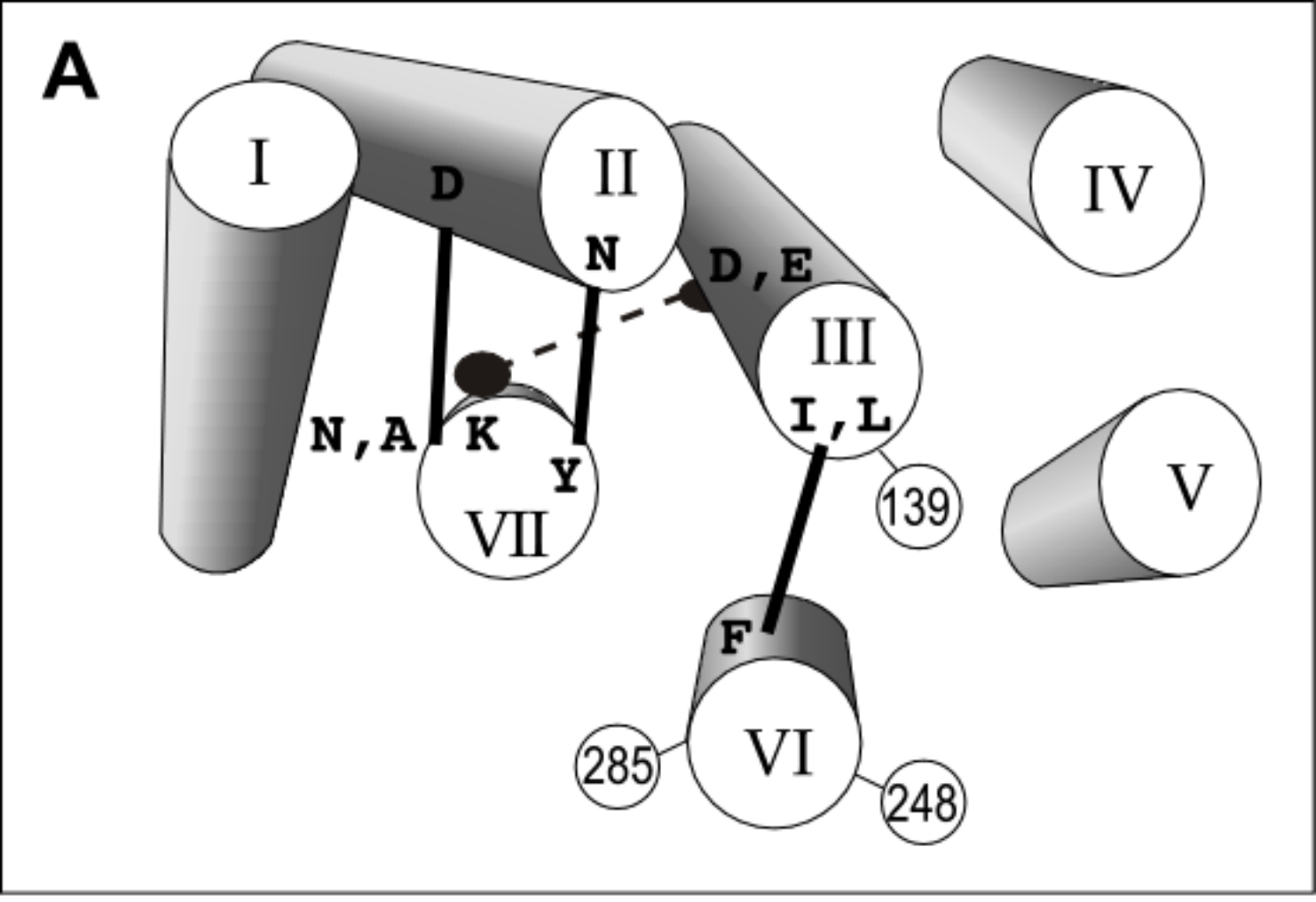

LB1
S
S
LB1
LB2
LB1a
LB1b
LB1a
LB1b
2
3
1
1
3
2
LB2b
LB2a
LB2b
LB2a
CR
CR

Figure 4


A
I
II
III
IV
V
VI
VII
D
N
D, E
N, A
K
Y
I, L
F
139
285
248

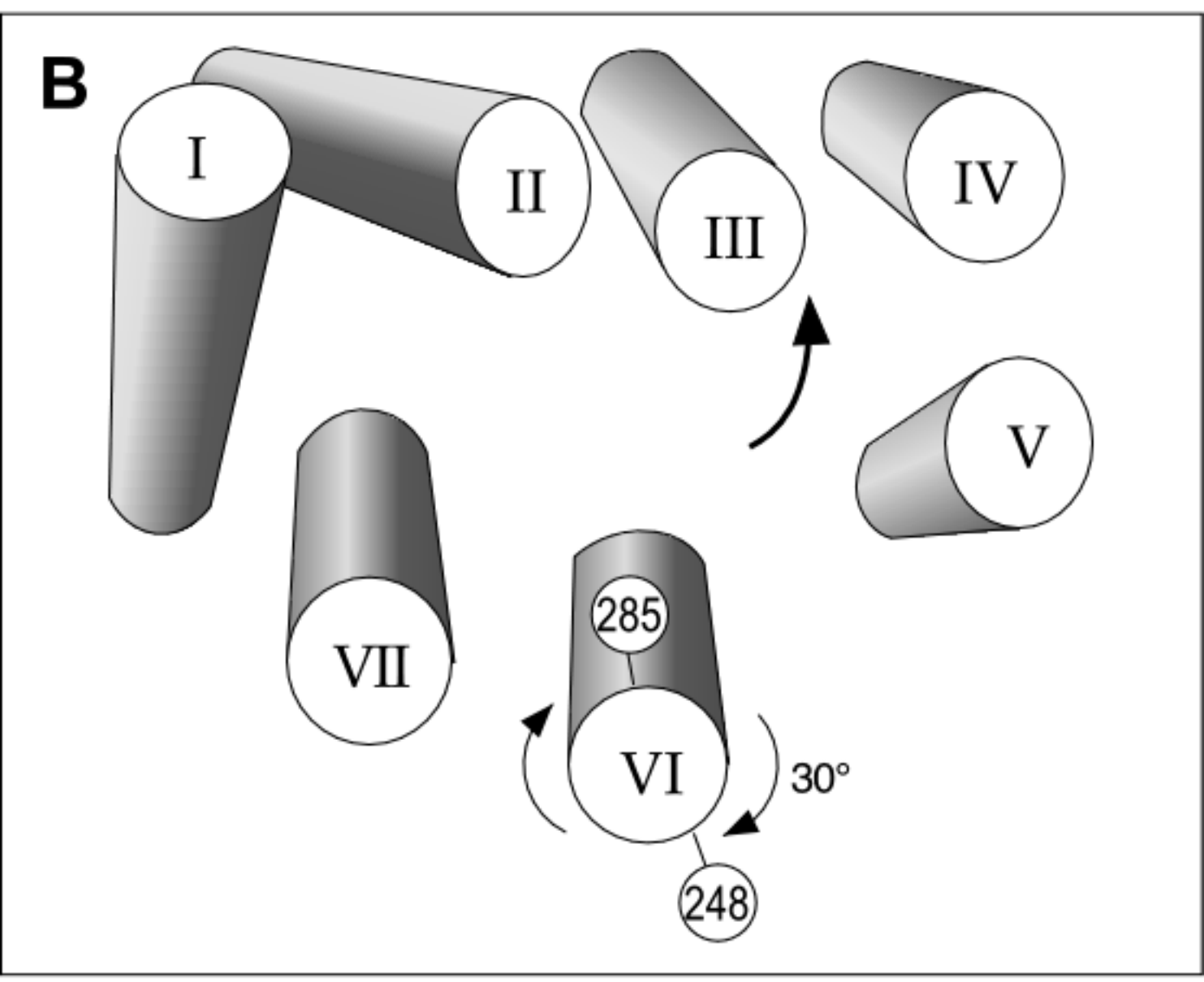

B
I
II
III
IV
V
VII
285
VI
30°
248

Figure 5

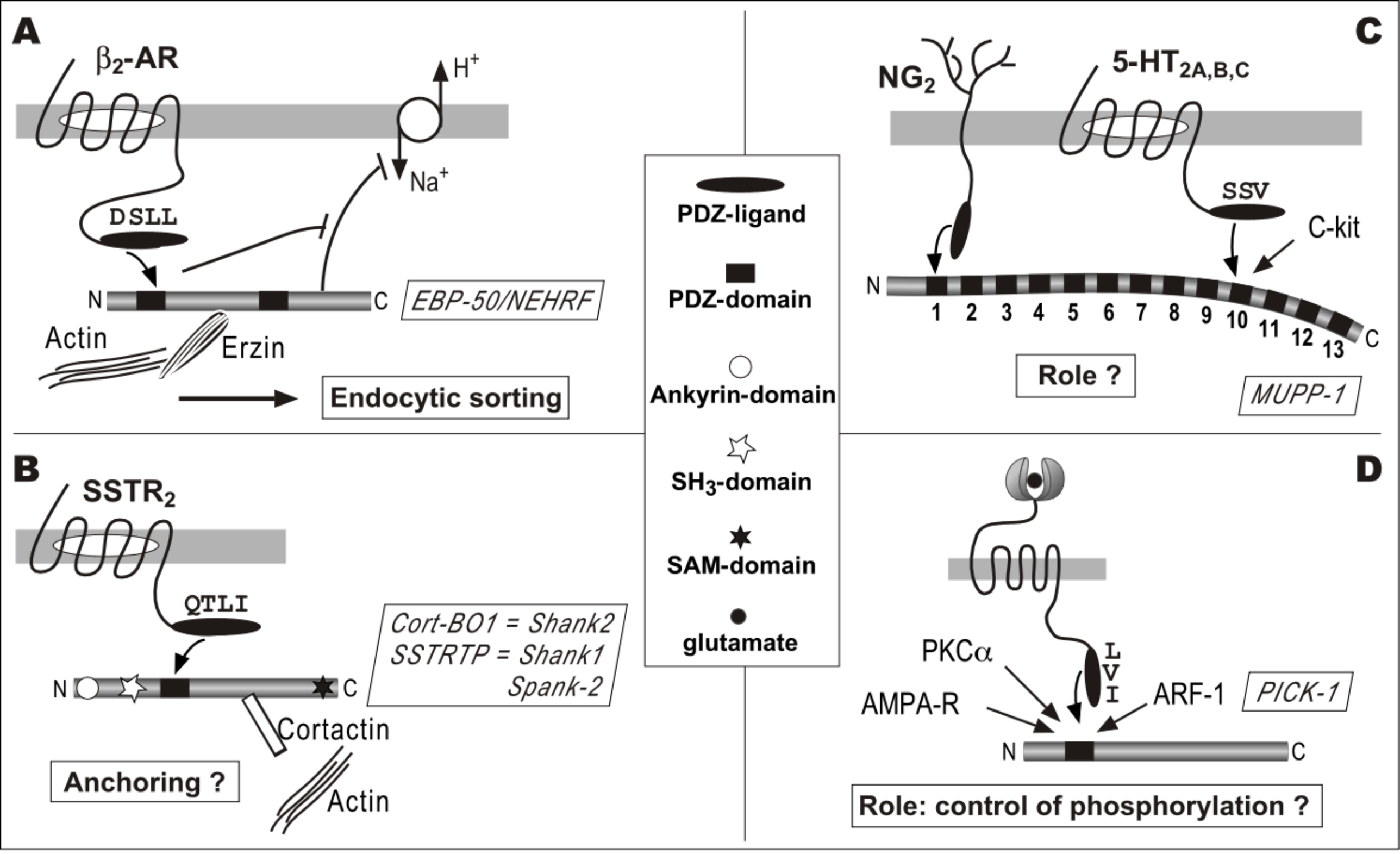

A
β2-AR
H+
Na+
DSLL
N
C
EBP-50/NEHRF
Actin
Erzin
Endocytic sorting
B
SSTR2
QTLI
Cort-BO1 = Shank2
SSTRTP = Shank1
Spank-2
N
C
Cortactin
Actin
Anchoring ?
PDZ-ligand
PDZ-domain
Ankyrin-domain
SH3-domain
SAM-domain
glutamate
C
NG2
5-HT2A,B,C
SSV
C-kit
N
1 2 3 4 5 6 7 8 9 10 11 12 13
C
Role ?
MUPP-1
D
PKCα
L
V
I
AMPA-R
ARF-1
PICK-1
N
C
Role: control of phosphorylation ?

Figure 6

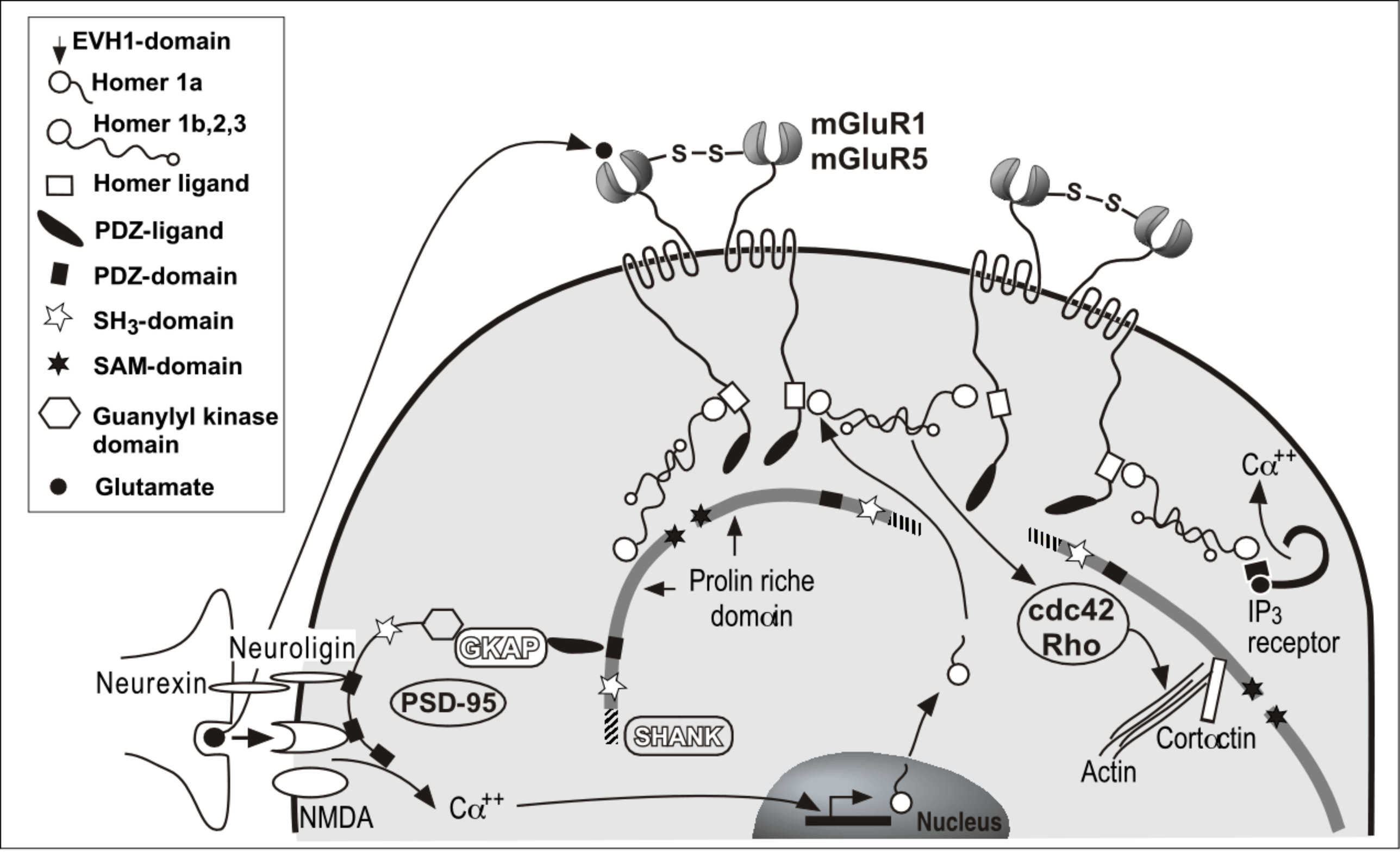

EVH1-domain
Homer 1a
Homer 1b,2,3
Homer ligand
PDZ-ligand
PDZ-domain
SH3-domain
SAM-domain
Guanylyl kinase domain
Glutamate
mGluR1
mGluR5
S–S
Prolin riche domain
Neuroligin
Neurexin
GKAP
PSD-95
SHANK
NMDA
Cα++
Nucleus
cdc42
Rho
IP3
receptor
Cortactin
Actin

Figure 7

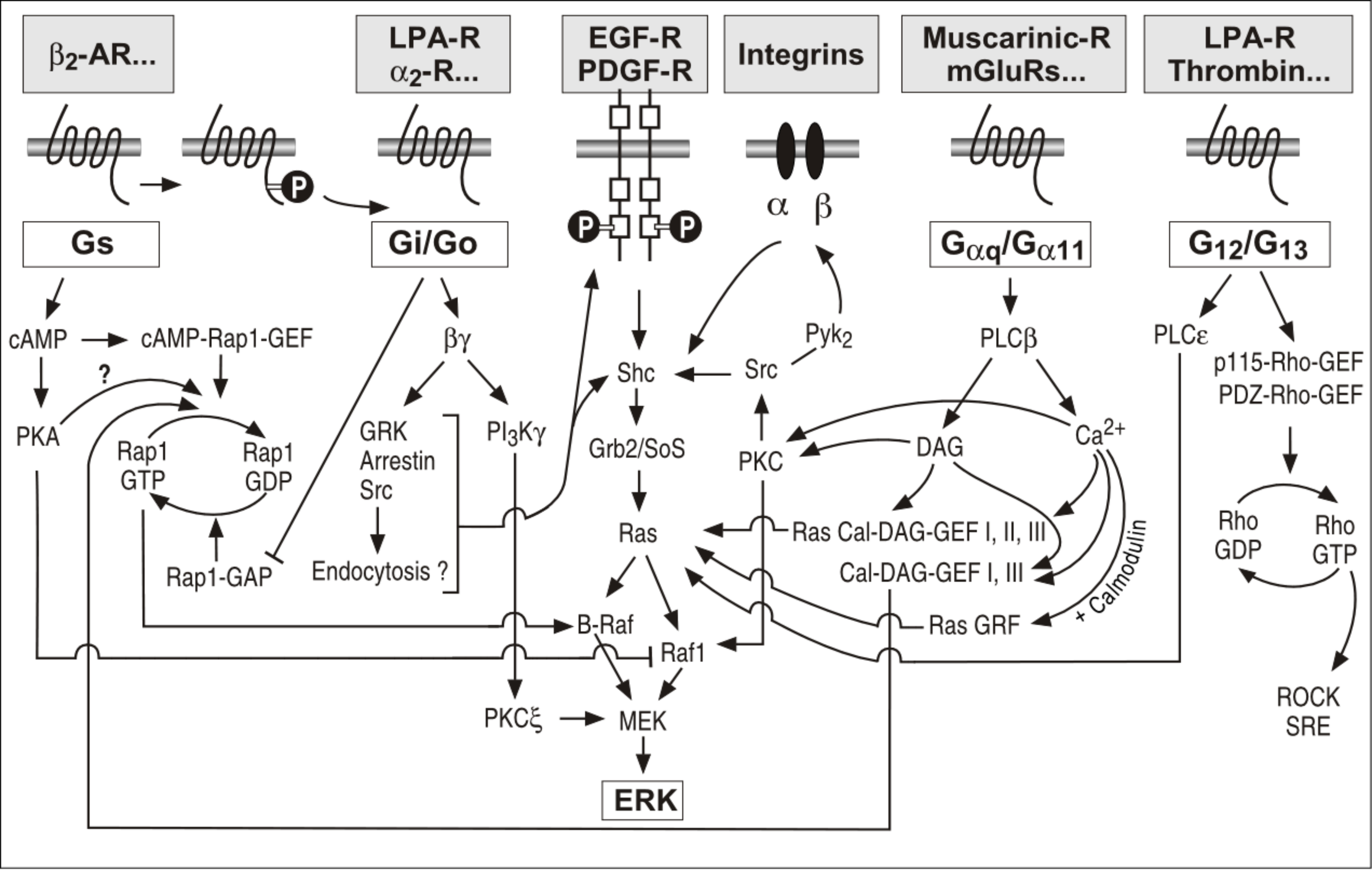

β2-AR...
LPA-R
α2-R...
EGF-R
PDGF-R
Integrins
Muscarinic-R
mGluRs...
LPA-R
Thrombin...
P
Gs
Gi/Go
α β
Gαq/Gα11
G12/G13
cAMP
cAMP-Rap1-GEF
?
βγ
Shc
Src
Pyk2
PLCβ
PLCε
p115-Rho-GEF
PDZ-Rho-GEF
PKA
Rap1
GTP
Rap1
GDP
GRK
Arrestin
Src
PI3Kγ
Grb2/SoS
PKC
DAG
Ca2+
Ras
Ras Cal-DAG-GEF I, II, III
Cal-DAG-GEF I, III
+ Calmodulin
Ras GRF
Rho
GDP
Rho
GTP
Rap1-GAP
Endocytosis ?
B-Raf
Raf1
PKCξ
MEK
ERK
ROCK
SRE

Figure 8