PHARMACOLOGICAL REVIEWS

# Molecular Mechanisms and Therapeutical Implications of Intramembrane Receptor/Receptor Interactions among Heptahelical Receptors with Examples from the Striatopallidal GABA Neurons

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*Abstract*——The molecular basis for the known intramembrane receptor/receptor interactions among G protein-coupled receptors was postulated to be heteromerization based on receptor subtype-specific interactions between different types of receptor homomers. The discovery of $GABA_B$ heterodimers started this field rapidly followed by the discovery of heteromerization among isoreceptors of several G protein-coupled receptors such as $\delta/\kappa$ opioid receptors. Heteromerization was also discovered among distinct types of G protein-coupled receptors with the initial demonstration of somatostatin $SSTR_5$/dopamine $D_2$ and adenosine $A_1$/dopamine $D_1$ heteromeric receptor complexes. The functional meaning of these heteromeric complexes is to achieve direct or indirect (via adapter proteins) intramembrane receptor/receptor interactions in the complex. G protein-coupled receptors also form heteromeric complexes involving direct interactions with ion channel receptors, the best example being the $GABA_A$/dopamine $D_5$ receptor heteromerization, as well as with receptor tyrosine kinases and with receptor activity modulating proteins. As an example, adenosine, dopamine, and glutamate metabotropic receptor/receptor interactions in the striatopallidal GABA neurons are discussed as well as their relevance for Parkinson's disease, schizophrenia, and drug dependence. The heterodimer is only one type of heteromeric complex, and the evidence is equally compatible with the existence of higher order heteromeric complexes, where also adapter proteins such as homer proteins and scaffolding proteins can exist. These complexes may assist in the process of linking G protein-coupled receptors and ion channel receptors together in a receptor mosaic that may have special integrative value and may constitute the molecular basis for some forms of learning and memory.

# I. Experimental Evidence on Protein/Protein Interactions Involving G Protein-Coupled Receptors in the Central Nervous System

## A. Early Indications for Intramembrane Receptor/Receptor Interactions Involving G Protein-Coupled Receptors

Emerging evidence shows that G protein-coupled receptors (GPCR[2]) can form homo- and heteromers (Bou-

[2] Abbreviations: GPCR, G protein-coupled receptor; ADA, adenosine deaminase; ADM, adrenomedullin; AT, angiotensin; BRET, bioluminescence resonance energy transfer; CaMK, $Ca^{2+}$/calmodulin-dependent protein kinases; CCK, cholecystokinin; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; CRE, cAMP response element; CREB, cAMP response element-binding protein; CRL, calcitonin receptor-like; DARPP-32, dopamine and cyclic adenosine $3',5'$-monophosphate-regulated phosphoprotein, 32 kDa; EGF, epidermal growth factor; FRET, fluorescence resonance energy transfer; Gal, galanin; GRK, GPCR kinase; GST, glutathione S-transferase; GTP$\gamma$S, guanosine $5'$-$O$-(3-thio)triphosphate; HEK, human embryonic kidney; 5-HT, 5-hydroxytryptamine (serotonin); $IP_3$, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular-regulated kinase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NK, neurokinin; NMDA, $N$-methyl-D-aspartate; NPY, neuropeptide Y; NT, neurotensin; PAGE, polyacrylamide gel electrophoresis; PAR, protease-activated receptor; PCP, phencyclidine; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PP-1, protein phosphatase-1; PSD,

vier, 2001; Marshall, 2001). It all began in 1979–1980 in search of an explanation of where all the recently discovered neuropeptides in the brain could integrate their messages with those of classical transmitters such as the monoamines. Luigi F. Agnati and Kjell Fuxe postulated that an intramembrane interaction between neuropeptide and monoamine receptors could be involved. The first observations were published in 1980 (Agnati et al., 1980), showing that substance P could modulate the high-affinity serotonin binding sites in spinal cord membrane preparations using biochemical binding techniques (also, see Agnati et al., 1983b). The same year, an interesting paper was published by Maggi et al. (1980) showing that the $\beta$ adrenergic receptor agonist isoproterenol could increase $\alpha_2$ adrenergic receptor binding in cortical slices, supporting the concept of intramembrane receptor/receptor interactions of GPCR, in this case among isoreceptors. Subsequently, in 1981 the existence of cholecystokinin (CCK) receptor/dopamine $D_2$ receptor interactions using biochemical binding techniques was indicated since CCK-8 could modulate the dopamine $D_2$ receptor antagonist and agonist binding sites in striatal membrane preparations (Fuxe et al., 1981, 1983b; Agnati et al., 1983a,b, 1985). Further evidence for receptor/receptor interactions came in 1982 from Lundberg, Bartfai, and colleagues (Lundberg et al., 1982) and from Zarbin and colleagues (Zarbin et al., 1982). Using the same type of approach, a large number of papers were published in 1983 that suggested the existence of intramembrane receptor/receptor interactions between different GPCR (Fuxe et al., 1983b; Agnati et al., 1984; Fuxe and Agnati, 1985). Those included neurotensin (NT receptor)/$D_2$ (Agnati et al., 1983c; Nemeroff, 1986; Von Euler and Fuxe, 1987; Von Euler, 1991), $CCK_B$/serotonin 5-$HT_2$ (Agnati et al., 1983a, 1985), vasoactive intestinal peptide (VIP) receptor/serotonin 5-$HT_1$ (Rostene et al., 1983a,b), neuropeptide Y (NPY) receptor/$\alpha_2$ adrenergic (Agnati et al., 1983d) and neurokinin $NK_1$/5-$HT_1$ receptor/receptor interactions (Agnati et al., 1983e). Subsequently in the 1980s, indications for glutamate receptor/$D_2$ receptor interactions (Fuxe et al., 1984) were obtained in striatal membrane preparations after earlier observations had indicated the existence of interactions at the membrane level among glutamate receptor subtypes (Fuxe et al., 1983c). This early research led to the following postulation in the opening address of Fuxe and Agnati at the International Wenner-Gren Symposium on receptor/receptor interactions in 1986 (Fuxe and Agnati, 1987) ". . . we will find out that some sophisticated elaborations are performed at the membrane level, via interactions within and among different classes of macromolecules (such as receptors, ion pumps, ion channels)" (also, see Agnati et al., 1988). In 1988,

postsynaptic density protein; RAMP, receptor activity-modifying protein; RTK, receptor tyrosine kinase; SST, somatostatin; TCF/Elk-1, ternary complex factor/Elk-1; TM, transmembrane; trunc, truncated; VT, volume transmission; WT, wiring transmission.

evidence for galanin (Gal) receptor/serotonin 5-$HT_{1A}$ receptor interactions in limbic cortical membranes (Fuxe et al., 1988a), as well as for angiotensin II receptor ($AT_1$)/$\alpha_2$ adrenergic receptor interactions (Fuxe et al., 1988b) in the medulla oblongata membrane preparations were obtained. In the early 1990s, adenosine $A_{2A}$ receptor/$D_2$ receptor interactions (Ferré et al., 1991d, 1993; Ferré and Fuxe, 1992) were demonstrated in striatal membrane preparations.

Thus, not only neuropeptide and monoamine receptors were involved in intramembrane receptor/receptor interactions but also certain types of glutamate and adenosine receptors (Agnati et al., 1986, 1990, 1993; Härfstrand et al., 1988; Tanganelli et al., 1989, 1990, 1993; Von Euler et al., 1989; Fuxe et al., 1990a–c, 1991, 1992a–c; Ferré et al., 1992, 1993b; Fior et al., 1993; Yang et al., 1994b). These results were all obtained at the recognition site of the receptors, using saturation and competition binding experiments. The modulation of binding could be shown as changes in $K_D$ and $B_{max}$ values (saturation analysis) and as $K_L$, $K_H$, and $R_H$ values (competition analysis) allowing a determination of modulation of the high- versus the low-affinity states of the receptor. An indication of an effect on the G protein-coupling and thus on the efficacy of the modulated receptor could be obtained by studying how, e.g., the modulator could control the GTP-induced disappearance of the high-affinity state of the receptor (reduction of the $R_H$ values). This would imply a G protein activation with formation of $G_\alpha$-GTP and $\beta\gamma$ dimers associated with a cross-regulation of the GPCR with a disappearance of the high-affinity state of the receptor.

In this period, the above work was extended to show multiple receptor/receptor interactions. Thus, evidence was obtained for a dopamine $D_1$ receptor involvement in the $CCK_B$ receptor/$D_2$ receptor interaction. Coactivation of $D_1$ and $D_2$ receptors led to an enhancement of the affinity of $D_2$ receptor agonist sites by CCK-8, instead of a reduced affinity of $D_2$ receptor agonist sites observed without $D_1$ receptor stimulation (Li et al., 1994a). These results are in line with the findings of Seeman et al. (1989) suggesting reciprocal interactions between $D_1$ and $D_2$ receptors in striatal homogenates. Thus, there may exist striatal nerve cell populations where intramembrane multiple $CCK_B$ receptor/$D_1$ receptor/$D_2$ receptor interactions can take place (Agnati et al., 1982).

In the early 1990s, evidence was also obtained that striatal NT receptors involved in the G protein-independent antagonistic regulation of striatal $D_2$ receptors (Von Euler et al., 1991) may represent a novel type of a high-affinity NT receptor. This was suggested in view of the rank order of potency found among COOH-terminal NT fragments, neuromedin N, and NT in this response versus the rank order of potency found at the cloned high-affinity NT receptors ($NT_1$ receptors) (Li et al., 1993a,b). These effects were stronger in striatal sections (Li et al., 1994b), and recently, the NT-induced reduc-

tion of $D_2$ receptor affinity in striatal sections has been found to be blocked by a $NT_1$-like antagonist (Diaz-Cabiale et al., 2002a).

In 1993 (Zoli et al., 1993), the hypothesis was introduced that the molecular mechanism for these large numbers of intramembrane receptor/receptor interactions among GPCR could be the formation of heteromeric complexes, the simplest being a heterodimer. This concept was based on the indication at the time that dimerization upon agonist activation may be a general phenomenon essential for receptor activation (Hollenberg, 1991), the best example being the dimerization of tyrosine kinase receptors (Schlessinger 1988, 2000; Helldin 1995). Thus, it was assumed that GPCR exist mainly as homodimers that interact with other types of homodimers to form heterodimers. The relative proportions of homo- and heterodimers would be determined by the concentrations of the two transmitters, the density of the two receptors and their distribution patterns, and the unique features of each receptor/receptor interaction (Zoli et al., 1993). In fact, early evidence obtained on purified GPCR by, e.g., Venter and Fraser (1983) and Conn et al. (1982) indicated that the functional GPCR were in a dimeric state. The same year that our review article appeared, the first evidence was published that GPCR can exist as dimers (Ng et al., 1993). Thus, the $5\text{-}HT_{1B}$ receptor in Sf9 cells was in the immunoblot analysis shown to exist as dimers and monomers. Finally, it should be mentioned that in 1982 we had introduced the receptor mosaic hypothesis of learning and memory based on the formation of membrane receptor clusters and thus of high order oligomeric receptor complexes (Agnati et al., 2002). It was postulated (Agnati et al., 1982; Zoli et al., 1993) that the formation and/or stabilization of the heteromeric complexes of GPCR could be enhanced by associated (adapter) proteins especially in the synaptic membranes. It must be noted that most of the GPCR are located in extrasynaptic membranes and therefore the potential heteromeric complexes discussed above may also be reached by volume transmission (VT) signals (Agnati and Fuxe, 2000).

Recent experimental data exist confirming this hypothesis, and they are described in this review (see Table 1). Despite all this novel experimental evidence, there are many questions regarding the molecular mechanism of receptor heteromerization and among them the mapping of the residues involved in the interaction in the case of direct interactions and the identification of scaffolding and adapter proteins in the case of indirect interactions. It should be noted that both types of interaction, direct and indirect, are likely to occur.

### B. G Protein-Coupled Receptors Homo- and Heteromerization

*1. Homomerization of G Protein-Coupled Receptors.* A number of new approaches made it possible to obtain convincing evidence for the existence of ho-

momers of many types of GPCR. Those include complementary chimeras, coimmunoprecipitation with differentially epitope-tagged receptors, the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), often in combination with covalent cross-linking, and finally biophysical methods, namely bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET).

In the mid 1980s, evidence for dimerization was obtained with experiments with photo-affinity labeling, radiation inactivation, cross-linking, and hydrodynamic analysis (Fraser and Venter, 1982; Avissar et al., 1983; Herberg et al., 1984; Peterson et al., 1986). However, it was the work of Maggio, Wess, and colleagues (Maggio et al., 1993) that offered strong indications for the existence of dimers, with the transcomplementation results obtained using cholinergic $M_3$ muscarinic receptor/$\alpha_2$ adrenergic receptor chimeras. In fact, when a chimera consisting of 5TM regions of one receptor and two of the other was expressed, there was a lack of ligand binding and of function that was recovered after coexpression of the two types of chimeras. This finding had a major impact, and the interpretation that the functional transcomplementation was caused by intermolecular interactions leading to the formation of a dimeric complex was early on accepted (Monnot et al., 1996). The early work in the mid-1970s demonstrating negative cooperativity in $\beta$-adrenergic receptors, opening up the possibility of dimer formation should also be mentioned (Limbird et al., 1975; Limbird and Lefkowitz, 1976). More recently, structural implications for V2 vasopressin receptor oligomerization have been given by Schultz et al. (2000) from functional reconstitution studies.

The SDS-PAGE approach provided one of the first demonstrations of GPCR dimers, namely of $5\text{-}HT_{1B}$, $D_1$, and $D_2$ receptor homomers (Ng et al., 1993, 1994a,b, 1996; George et al., 1998; Zawarynski et al., 1998). These observations are based on the fact that several homomers are resistant to the denaturation properties of SDS. Thus, it was possible early on for George, O'Dowd, and colleagues to observe upon SDS-PAGE that the $5\text{-}HT_{1B}$, $D_1$, and $D_2$ receptors expressed in cell lines formed molecular species not only corresponding to monomers but also to dimers (Ng et al., 1993, 1994a,b; Lee et al., 2000). These dimeric and even higher order oligomeric complexes were not caused by glycosylation of monomers nor to the presence of G proteins (Lee et al., 2000). Treatment with covalent cross-linkers before solubilization increased the proportion of dimeric complexes and facilitated the demonstration of dimers in immunoblots (Hebert et al., 1996; Romano et al., 1996). It should be considered that monomers at least in some cases could represent the disruption of dimers or higher oligomeric complexes (Lee et al., 2000). Also by using the SDS-PAGE strategy, the existence of $D_2$ and $A_1$ receptors homomers was demonstrated, for the first time, in brain tissue, showing their existence in situ and not only

TABLE 1
*Intramembrane receptor-receptor interactions via heteromerization*

| Type of Receptors Involved | Possible Mechanism for Interaction | Changes at Recognition Level | Changes at Signaling Level | Changes in Receptor Trafficking | Possible Therapeutic Implications |
|---|---|---|---|---|---|
| $GABA_BR1/GABA_BR2$ | Coiled-coil interaction at COOH terminus (heterodimers are preferred) | Increased potency of agonists (R1, R2) | Essential for signaling (R1, R2) | Essential for cell surface expression (R1) | Anticonvulsive therapy |
| $\delta$ opioid/$\kappa$ opioid | Direct interaction (heterodimers are preferred) | High affinity for unselective ligands | Synergistic activation of MAPK | Reduced $\delta$ R internalization | Pain relief, drug dependence |
| $\delta$ opioid/$\mu$ opioid | Direct interaction (heterodimers are preferred) | Increased affinity for certain enk peptides | Altered selection of G protein (e.g., $G_z$) | Unknown | Pain relief, drug dependence |
| $5\text{-}HT_{1B}/5\text{-}HT_{1D}$ | Direct interaction (heterodimers are preferred) | Unknown | Unknown | Unknown | Antidepressant treatment |
| $D_2/D_3$ | Direct interaction | No clear-cut changes | Increased coupling of $D_3$ to AC | Unknown | Antipsychotic treatment |
| $SSTR_5/SSTR_1$ | Direct interaction | Up-regulation of $SSTR_1$ agonist binding | Sensitization of $SSTR_1$ after $SSTR_5$ activation | Unknown | Unknown |
| $SSTR_5/D_2$ | Agonist-dependent direct interaction | Positive reciprocal affinity regulation | Enhancement of signaling | Unknown | Antiparkinsonian treatment |
| $A_1/D_1$ | Agonist regulation of heteromerization: ADA dependency | Disappearance of $D_1$-R high affinity state | Desensitization of $D_1$-R after $A_1/D_1$ agonist treatment | $A_1$-induced coaggregation of $A_1$-R/$D_1$-R | Antiparkinsonian treatment drug dependence |
| $mGLU\text{-}R_{1\alpha}/A_1$ | Involvement of mGLU-$R_{1\alpha}$ COOH terminus: Homer dependency? | Unknown | Reciprocal enhancement of $Ca^{2+}$ signaling | Unknown | Neuroprotection |
| $P_{2Y1}/A_1$ | Direct interaction | Novel $A_1$-R binding pocket? | Altered selection of G protein | Unknown | Neuroprotection |
| $A_{2A}/D_2$ | Possible involvement of D2 5,6 TM domains and intracellular loop 3 | Reduced $D_2$-R affinity, especially high-affinity state | Reduced $D_2$-R $Ca^{2+}$ and cAMP signaling | Coaggregation, cointernalization, codesensitization | Antiparkinsonian, antipsychotic, antidyskinetic treatment |
| $A_{2A}/mGLU\text{-}R_5$ | Homer dependency? | Unknown | Synergism for c-Fos expression | Unknown | Antiparkinsonian, antipsychotic, antidyskinetic drug dependence treatment |
| $B_2/AT_1$ | Unknown (no adapter proteins) | Complex regulation of $AT_1$-R affinity | Increase in $AT_1$-R coupling to G protein | Altered receptor trafficking | Antihypertensive treatment |
| $D_5/GABA_A$ | COOH-terminal $\gamma_2$ intracellular loop 2, (agonist coactivation-dependent) | No changes in $D_5$-R binding | Reduction of $GABA_A$-dependent currents and of $D_5$-R signaling | Agonist induced cotrafficking | Antipsychotic treatment |
| $\beta_2/EGF\text{-}R$ | Multiprotein complex (involvement of $\beta$-arrestin) agonist dependency | Unknown | Transactivation of EGF-R | Favoring of clathrin-mediated endocytosis | Neuroprotection, neuroplasticity |

in cell lines where artificially high levels of receptors are expressed (Ciruela et al., 1995; Ng et al., 1996).

The coimmunoprecipitation approach was first described in the article by Bouvier and colleagues in 1996 (Hebert et al., 1996) on $\beta_2$ adrenergic receptors with coexpression of differentially tagged $\beta_2$ adrenergic receptors. This coimmunoprecipitation was taken as evidence for the existence of a $\beta_2$ adrenergic receptor dimer. Peptides corresponding to TM6 were found to disrupt the dimerization as well as receptor activation indicating a participation of the hydrophobic forces in the TM6 region in the dimerization interface (Hebert et al., 1996; Ng et al., 1996). The focus on the TM6 came from the work of Engelman et al. (Lemmon et al., 1992; Lemmon and Engelman, 1994), showing that dimerization is driven by specific interactions among TM $\alpha$-helices. A number of GPCR homomers have been demonstrated

with this approach, e.g., the metabotropic glutamate receptor (mGlu5) homomer, where the disulfide bridges between the large extracellular $NH_2$-terminal domains play a role in the formation of the homomer (Romano et al., 1996; Bouvier, 2001).

The techniques of BRET and FRET imply as close distances as 5 to 10 nM between donor and acceptor for energy transfer. In FRET both the donor and acceptor are fluorescent molecules, whereas for BRET the donor is bioluminescent and the acceptors fluorescent. These techniques have been very valuable in detecting dimers in living cells without the risk of solubilization artifacts (for details on methodology, see Bouvier, 2001). In 2000, the BRET approach could for the first time demonstrate homomerization of $\beta_2$ adrenergic receptors in living cells (Angers et al., 2000) independently, and at the same time, the FRET procedure revealed the homomerization

of the yeast $\alpha$ mating factor in living cells (Overton and Blumer, 2000). In 2001, using the FRET and BRET technologies, constitutive homo-oligomerization could be demonstrated for $\delta$ opioid receptors in intact cells (McVey et al., 2001). The same was also found to be true for the thyrotropin-releasing hormone (TRH) receptors using the BRET technique (Kroeger et al., 2001). With FRET technique, the somatostatin receptor subtypes have been shown to assemble, e.g., as homomers (Rocheville et al., 2000b) using differential epitope tagging and fluorescently labeled antibodies against the epitopes.

Together with results from coimmunoprecipitation experiments these results indicate that several, if not many, GPCR undergo constitutive homomerization, i.e., the basal state of the GPCR may be the dimer. It remains to be shown if the existence of constitutive dimers can help explain the constitutive, agonist independent, activity of several GPCR. We have, e.g., recently observed that $D_2$ receptor antagonists do not affect or even reduce the $D_2$ receptor clustering (increased by $D_2$ receptor agonists) in the basal state of CHO cell lines expressing human $D_{2L}$ receptors on their surface. Such an effect of the $D_2$ receptor clustering by a $D_2$ receptor antagonist in the absence of a dopamine receptor agonist could at least in part explain an inverse $D_2$ agonist activity of the $D_2$ antagonist (L. F. Agnati, S. Ferré, R. Franco, and K. Fuxe, unpublished data). Therefore, the action of the agonist at GPCR may sometimes be to produce a conformational change in the basal homomer leading to the development of the active state. Again it should be emphasized that the basal state instead sometimes may be represented by a monomeric or higher oligomeric form of the GPCR.

*2. Heteromeric Complexes Involving G Protein-Coupled Receptors.* The existence of heteromers of GPCR was postulated in the 1993 Molecular Neurobiology review article (Zoli et al., 1993) to give a molecular basis for the large amount of evidence we had obtained on the existence of receptor/receptor interactions among GPCR (see above). It was therefore inspiring when the first evidence of the existence of a GPCR heteromer, namely the $GABA_B$ heteromer, came in 1998/99 (Jones et al., 1998; Kaupman et al., 1998; White et al., 1998; Kuner et al., 1999; Marshall et al., 1999; Ng et al., 1999). The field of heteromerization has been excellently summarized by Marshall (2001) and by Bouvier (2001). Here some key examples will be discussed together with the evidence suggesting that the heterodimerizaton and hetero-oligomerization may be the main molecular basis for the previously observed receptor/receptor interactions among GPCR (Fuxe and Agnati, 1985, 1987; Agnati et al., 1993; Zoli et al., 1993; Fuxe et al., 1995, 1996, 1998; Ferré et al., 1997). The functional relevance, the pathological implications and the relevance for new drug development (see Agnati et al., 1986, 1990; Fuxe et al.,

1989, 1998) of certain selected receptor heteromers are also discussed.

One question that arises out of the recent reports is how to understand the architecture of cross-talk among heteromeric complexes. It is interesting to ask, for instance, how the strongly antagonistic $A_{2A}/D_2$ intramembrane receptor/receptor interaction through its heteromeric complex (Hillion et al., 2002) becomes integrated with the strongly facilitatory $SSTR5/D_2$ intramembrane receptor/receptor interaction through its heteromeric complex (Rocheville et al., 2000a). Do these heteromeric complexes directly interact? Are they part of the same molecular circuits of the same striatal nerve cell or are they independently located in different membrane domains of the same cell or even in different striatal nerve cell populations? These will be important studies to perform in order to understand the integration of transmitter signals in the striatum and in general in the brain. How do the various heteromeric complexes of GPCR interact at membrane level and downstream at intracytoplasmatic level? How are these two levels of interactions integrated? These are fundamental questions to be answered and a possible heuristic frame to tackle these questions is discussed below (*Section II.C.*).

*a. The $GABA_B$ Receptor Heterodimer.* The first evidence that two subtypes of $GABA_B$ receptors, $GABA_BR1$ and $GABA_BR2$, undergo heterodimerization and that this process is essential for the cell surface expression of the functional receptor was given in three studies published simultaneously in December 1998 (Kaupmann et al., 1998; Jones et al., 1998; White et al., 1998). This was also demonstrated by Kuner et al. and Ng et al. in January 1999 (see also Gordon et al., 1999; Mitrovic et al., 2000). The physiological relevance of these findings is supported by the demonstration of coimmunoprecipitation in cerebral cortex membranes of $GABA_BR1$ (a or b) with $GABA_BR2$ proteins, of their colocalization in dendritic spines (Kaupmann et al., 1998) and of substantial degree of coexpression of $GABA_BR1$ and $GABA_BR2$ mRNA levels in many nerve cell populations (Kuner et al., 1999). Changes also occurred at the recognition site level as a result of the heterodimerization, since the potency of agonists and partial agonists became increased (Kaupmann et al., 1988).

The most dramatic change is, however, that the formed $GABA_B$ receptor heteromer, unlike its monomeric components, can become functional and couple to the G protein leading to regulation of the inwardly rectifying $K^+$ channels, the $Ca^{2+}$ channels, and adenylyl cyclase (Alger and Nicoll, 1979; Bettler et al., 1998). Thus, it seems as if the predominant native $GABA_B$ receptor is the $GABA_B$ receptor heteromer. Evidence has been presented that the COOH-terminal domain is involved in the formation of this heterodimer by a coiled-coil interaction (Kammerer et al., 1999; Kuner et al., 1999).

In 2000, the important observation was made that the coiled-coil interaction at the COOH-terminal domain blocks a retention motif for the endoplasmic reticulum of the GABA$_B$R1 receptor (Margeta-Mitrovic et al., 2000). The masking of this motif allows the heterodimers to travel to the cell surface. However, even if in the mutant GABA$_B$R1 the retention motif had been removed and this mutant receptor could be expressed on the cell surface, it still remained functionally inactive underlining a probable crucial role of GABA$_B$ receptor heterodimerization in signaling.

These important findings give a clear example of the functional relevance of intramembrane receptor/receptor interactions through heteromerization, namely in receptor trafficking, including receptor maturation and receptor cell surface expression, and in receptor signaling, i.e., in G protein coupling and in increased binding potency of agonists and partial agonists, in line with previous work on receptor/receptor interactions (see Fuxe and Agnati, 1985, 1987; Zoli et al., 1993).

It is well known that GABA$_B$ receptors play a distinct role in modulating the neuronal networks, and agonist drugs acting on these receptors appear to have inter alia anticonvulsive and anxiolytic properties. It is therefore of substantial interest that the anticonvulsive compound gabapentin is a selective agonist at the GABA$_B$R1a/GABA$_B$R2 heteromer (Ng et al., 2001). This is an example of how the molecular composition of the heteromer determines its pharmacological profile and gives rise to a novel GABA$_B$ receptor agonist selective for a certain type of GABA$_B$ receptor heteromer dependent on the splice variant involved. In this study, these results were correlated with a selective ability to increase postsynaptic GABA$_B$ receptor signaling (opening of inwardly rectifying K$^+$ channels) without altering GABA transmission at the presynaptic level (Ng et al., 2001). This novel type of pharmacological selectivity based on unique heteromers may therefore have considerable potential for drug development. It serves to show the pharmacological relevance of intramembrane receptor/receptor interactions that may give rise to novel receptor subtypes with a unique pharmacology based on the composition of the heteromer formed changing the biochemical characteristics of the binding pocket of the receptor.

*b. Heteromerization of δ and κ Opioid Receptors and of μ and δ Opioid Receptors.* After the discovery of the GABA$_B$ receptor heterodimers the discovery of the δ/κ opioid receptor dimer came next (Jordan and Devi 1999; Jordan et al., 2000) followed by another interesting paper on μ/δ opioid receptor heteromerization by George et al., (2000). In this case, however, the two receptors of the heterodimer were functional on their own and could reach the cell surface without heteromerization with another opioid receptor subtype.

These discoveries were in a way expected, since early work had given indications for the existence of a μ/δ opioid receptor complex (Rothmann et al., 1988; Schof-

felmeer et al., 1990). Furthermore, the opiate receptor field had for some time discussed the possibility that heterodimerization among the cloned μ, δ, and κ opioid receptors could explain the existence of more than three opioid receptor subtypes as characterized pharmacologically (Kieffer, 1999). The δ/κ opioid heteromer (Jordan and Devi, 1999; Jordan et al., 2000) was shown to have a unique pharmacology with high affinity for rather unselective ligands but very little affinity for δ- and κ-selective compounds. Nevertheless, the selective δ and κ agonists, when given at the same time, bound synergistically to the heterodimers associated with a synergistic activation of the mitogen-activated protein kinase (MAPK). Thus, a novel subtype of opioid receptor binding pocket may have appeared through this receptor/receptor interaction via δ/κ opioid receptor heterodimerization. Also, the δ and κ heteromer had consequences for the agonist-induced internalization of the δ opioid receptor, which became reduced. Thus, another functional role of this intramembrane receptor/receptor interaction through heteromerization may be the control of receptor internalization.

The μ/δ opioid receptor heteromer, demonstrated by George et al. (2000), also showed changes in the pharmacological properties at the recognition site with reduced affinity for selective agonists and increased affinity for certain enkephalin peptides. Can in fact the μ/δ heteromer be the target of distinct enkephalin peptides? Of special interest was the demonstration that the μ/δ heterodimer, unlike the μ and δ receptors when expressed alone, could become coupled to G proteins resistant to pertussis toxin, like G$_Z$. Thus, the G protein coupling has become markedly altered in the heteromer. In fact, the major function of this intramembrane receptor/receptor interaction based on μ/δ heteromerization may be a change in the selection of G protein coupling involving a conformational change in the G protein interface of the μ/δ receptor heteromer. The other functional change is altered binding properties of the recognition site seen as a novel pattern of ligand binding based on affinity changes; a novel binding pocket seems to have appeared (see Levac et al., 2002).

*c. The Serotonin 5-HT$_{1D}$/5-HT$_{1B}$ Receptor Heteromer.* This is an interesting demonstration of how two receptor subtypes of the type A receptor family (rhodopsin-like GPCR) when coexpressed preferentially form heteromers (Xie et al., 1999) without homomers. In contrast, when the two receptor subtypes were expressed alone homomers were formed. It is of substantial interest that the two receptor subtypes when coexpressed prefer the heteromer, since it indicates that at least in some cases there is a markedly displaced equilibrium between homomers and heteromers, since the heteromer is so clearly preferred. In this example the formation of the heteromer was not associated with a change in the binding pocket, and the functional relevance of this receptor/receptor interaction still remains to be determined.

Based on their expression patterns in the brain it seems as if they form different types of complexes in the brain, namely 5-HT$_{1B}$ receptor and 5-HT$_{1D}$ receptor homomers and 5-HT$_{1B}$/5-HT$_{1D}$ receptor heteromers.

*d. The Dopamine D$_2$/D$_3$ Receptor Heteromer.* The D$_2$ and D$_3$ receptors are known to exist as monomers and homomers (Nimchinsky et al., 1997; see Lee et al., 2000). In 2001, the evidence also came that D$_2$ and D$_3$ receptors can form heteromers with unique functional properties (Scarselli et al., 2001). Coimmunoprecipitation experiments using differentially tagged D$_2$ and D$_3$ receptors showed that D$_2$ and D$_3$ receptors in HEK-293 cells can form a heteromeric complex. Furthermore, they were able to demonstrate that heterologous cotransfected dopamine receptor fragments [D$_2$ trunc (TM1–5)/D$_3$ tail (TM6–7); D$_3$ trunc (TM1–5)/D$_2$ tail (TM6–7)] could form functional dopamine receptors that bound dopamine agonists and antagonists with a different pharmacological profile compared with native D$_2$ and D$_3$ receptors, with the highest affinity of all being found with the D$_3$ trunc/D$_2$ tail fragment combination. Thus, split D$_2$/D$_3$ heteromers may be formed through the domain-swapping mechanism as proposed by Gouldson, Reynolds, and colleagues (Gouldson et al., 1998, 2000) based inter alia on demonstrations of functional complementation between chimeras of $\alpha_2$ adrenergic and M3 muscarinic receptors (Maggio et al., 1993).

In agreement with the formation of D$_2$/D$_3$ heteromers in cells, these types of D$_2$ and D$_3$ receptor fragments, when coexpressed with native D$_2$ and D$_3$ receptors, reduced the expression of native dopamine receptors indicating fragment/native receptor complex formation. However, not only are D$_2$ trunc/D$_3$ tail and D$_3$ trunc/D$_2$ tail receptors able to bind ligands but they can also couple in an inhibitory way to adenylyl cyclase and to the same extent as the native D$_2$ receptor. It is also of substantial interest that the D$_3$ receptor under conditions in which it cannot inhibit adenylate cyclase VI (Robinsson and Caron, 1997) can develop such a coupling by cotransfection with D$_2$ receptors. It is therefore possible that in the D$_2$/D$_3$ heteromeric receptor complex formed, the D$_2$ receptor can make possible the G protein coupling of the D$_3$ receptor to adenylyl cyclase VI. Alternatively, the D$_3$ receptor binding pocket upon activation by D$_3$ agonists can, through conformational changes, transfer the D$_2$ pocket of the heteromer into an activated state, leading to G$_i$ activation and adenylyl cyclase VI inhibition. Finally, in the case of adenylyl cyclase V activity, the coexpression of D$_2$ and D$_3$ receptors even resulted in an increased potency of the D$_3$ receptor agonist to inhibit this adenylyl cyclase compared with wild-type D$_2$ receptors when activated by D$_2$ agonists. One function of the D$_2$/D$_3$ heteromeric receptor complex may therefore be to allow a stronger inhibitory coupling of the D$_3$ receptors to adenylyl cyclase.

Colocalization of D$_2$ and D$_3$ receptors has been demonstrated in nerve cells of the basal ganglia (Le Moine and Bloch, 1996; Gurevich and Joyce, 1999) showing that there is the potential to form functional D$_2$/D$_3$ heteromers also in vivo.

*e. The Somatostatin SSTR$_5$/SSTR$_1$ Receptor Heteromer.* The study by Rocheville et al. (2000b) gives a fine illustration of intramembrane receptor/receptor interactions and their functional relevance and of the relationship of monomers, homomers, and heteromers among five somatostatin receptor subtypes. Using FRET analysis, the human somatostatin receptor subtype SSTR$_5$ was shown to exist as a monomer in the basal state, which upon agonist activation was converted into a homomer. The data suggested that the agonist-induced dimerization of SSTR$_5$ receptors was essential for signaling. Agonist-induced heteromerization of SSTR$_5$ and SSTR$_1$ receptors could also be demonstrated, which appeared to be subtype specific.

The suggestion was made that the reported high level of basal homomer expression of GPCR could be due to receptor overexpression. The intramembrane SSTR$_5$ and SSTR$_1$ receptor/receptor interactions through heteromerization was shown to have important functional consequences for the participating receptors. Besides the changes in agonist affinity that usually develop upon changes in the oligomeric state, marked alterations in agonist-dependent internalization and an up-regulation of SSTR$_1$ receptors occurred through formation of a heteromer with SSTR$_5$ receptors. Thus, the SSTR$_1$ receptor only underwent agonist-induced internalization as a heterodimer with the SSTR$_5$ receptor. Furthermore, the heteromerization allowed a somatostatin receptor agonist (not binding to SSTR$_1$) to induce up-regulation of agonist binding at the SSTR$_1$ receptor. SSTR$_5$ receptor signaling via its G protein is probably not involved in this response of the heteromer, since the COOH-terminal tail of the SSTR$_5$ receptor had been deleted abolishing adenylyl cyclase regulation. These results are of substantial interest since in this way the desensitization of activated somatostatin receptor subtypes can be compensated for by an up-regulation of the nonactivated somatostatin receptor subtypes, such as SSTR$_1$, made possible through the heteromerization. Thus, another functional meaning of intramembrane receptor/receptor interactions via heteromerization may be the sensitization of one isoreceptor as the other isoreceptor of the heteromer undergoes desensitization.

*f. The Somatostatin SSTR$_5$ and Dopamine D$_2$ Heteromeric Receptor Complex.* The discovery of this intramembrane receptor/receptor interaction through hetero-oligomerization gave a novel way to understand the well known somatostatin/dopamine interactions in the brain involved, e.g., in the control of motor activity (Cohn and Cohn, 1975; Havlicek et al., 1976; Kastin et al., 1978; Chneiweiss et al., 1985; Glowinski and Premont, 1985; Martin-Iverson et al., 1986; Leblanc et al., 1988; Izquierdo-Claros et al., 1997; Rodriguez-Sanchez et al., 1997). In this case the hetero-oligomerization in-

volved distinct GPCR and not isoreceptors having the same or similar endogenous ligands. It was by means of photobleaching FRET microscopy that the direct $SSTR_5/D_2$ receptor/receptor interaction could be determined, and oligomerization was hardly observed in the basal state but only after treatment with either agonist. Simultaneous treatment with the two types of agonists together had no further action (Rocheville et al., 2000a). It is known that $D_2$ receptor homomers exist in the basal state (Lee et al., 2000), and somatostatin homomers are induced by somatostatin receptor agonists (see above). It remains to be determined whether heterodimers are formed or larger oligomeric complexes in the case of the $SSTR_5/D_2$ heteromerization.

The functional meaning of this direct intramembrane receptor/receptor interaction appeared to be severalfold (Rocheville et al., 2000a). First, the binding pocket of $SSTR_5$ receptor was markedly altered, since a 30-fold increase in affinity was found upon $D_2$ receptor agonist activation, whereas $D_2$ receptor antagonists reduced the affinity of $SSTR_5$ receptors for the somatostatin agonist SST-14. Thus, different conformational states (agonist-antagonist states) of the $D_2$ receptor have a substantial modulatory action on the binding pocket of the $SSTR_5$. The interaction at the recognition site level was also reciprocal since the somatostatin agonist enhanced the affinity of the $D_2$ receptor for antagonists. Second, the G protein coupling of the $SSTR_5$ receptor was enhanced by the $D_2$ receptor activation, since the reduction of $SSTR_5$ receptor agonist binding by GTP$\gamma$S was enhanced by the $D_2$ agonist. Furthermore, the inhibitory responses on cAMP accumulation were significantly enhanced by simultaneous agonist treatments, emphasizing the enhancement of the functional activity through the hetero-oligomer formed and the associated conformational changes induced by agonists in this complex. In fact, these functional changes in the intramembrane receptor/receptor interaction induced by $SSTR_5$ and $D_2$ receptor agonists may explain the increased somatostatin- and $D_2$ receptor-mediated neurotransmission found in vivo after somatostatin or dopamine agonist treatments.

In this study, Rocheville et al. (2000a) also used the mutant $\Delta$ 318-$SSTR_5$ receptor, with a COOH-terminal tail deletion. This mutant $SSTR_5$ receptor had been previously shown to bind somatostatin agonists with unchanged affinity, but different than the wild-type $SSTR_5$ receptor, it is not able to produce inhibition of forskolin-induced cAMP accumulation (Hukovic et al., 1998). The interesting finding was that cotransfection with $D_2$ receptors and mutant $\Delta$ 318-$SSTR_5$ receptors could restore the somatostatin agonist signaling to the adenylate cyclase provided the $D_2$ recognition site was not blocked by a $D_2$ antagonist. These observations can be explained by the formation of a hetero-oligomer in the CHO-k cells used, in which the $SSTR_5$ binding pocket when activated by agonists can signal via a conformational change in the dopamine $D_2$ binding pocket. This would lead to a

coupling of the $D_2$ recognition site to the $G_i$ protein followed by its activation and subsequent inhibition of adenylate cyclase. This conformational change cannot occur when the $D_2$ binding pocket is in an antagonistic binding state. It indicates in fact that the $SSTR_5$ receptor can signal via a conformational change in the $D_2$ receptor similar to that produced by the $D_2$ agonist. In other words, a cross-activation of the $D_2$ receptor can occur in the absence of dopamine by a direct receptor/receptor interaction in the receptor interfaces of the hetero-oligomer. Thus, the activated SST receptor cannot only modulate the activated $D_2$ receptor/$G_i$ protein coupling to adenylate cyclase but also produce a constitutive activity of the $D_2$ receptor when it is not locked into an antagonistic state. From another perspective, it represents an example of how a mutant GPCR can rescue its signaling by activating another receptor coupled to the same type of G protein.

*g. The Adenosine $A_1$ and Dopamine $D_1$ Heteromeric Receptor Complex.* The article on $A_1/D_1$ heteromers came out a couple of months after the appearance of the $SST5/D_2$ receptor oligomer article and gives another example of heteromerization between distinct GPCR (Gines et al., 2000). A number of morphological and neurochemical observations indicate that adenosine $A_1$ and dopamine $D_1$ receptor/receptor interactions exist in the basal ganglia (Ferré et al., 1994b, 1996a,b, 1997; Fuxe et al., 1998, 2002; Franco et al., 2001) and colocalization of $A_1$ and $D_1$ receptors exists in primary cortical cultures (Gines et al., 2000). The article by Ginés et al. (2000) gives the first evidence that this receptor/receptor interaction can involve $A_1/D_1$ heteromeric receptor complexes since such complexes could be demonstrated in cotransfected $A_1/D_1$ fibroblast Ltk$^-$ cells by means of coimmunoprecipitation. Thus, the previously found $A_1$ receptor-induced uncoupling of the $D_1$ receptor, demonstrated as the $A_1$ receptor-induced disappearance of the high-affinity $D_1$ receptor agonist binding sites in membrane preparations (Ferré et al., 1994b, 1998; Fuxe et al., 1998), could be the result of a physical interaction of the $A_1$ receptor with the $D_1$ receptor in this heteromeric complex, leading to an uncoupling of the $D_1$ receptor to its $G_s$-like protein in this functionally interacting heteromeric complex. The coimmunoprecipitation analysis demonstrates its existence already in the basal state and the specificity by the failure to show $A_1/D_2$ receptor heteromerization in $A_1/D_2$ receptor cotransfected fibroblast cells.

However, $A_1/D_1$ receptor heteromerization in the cotransfected fibroblast cells was strongly reduced by the $D_1$ receptor agonist treatment, showing an agonist dependence, and simultaneous $D_1$ and $A_1$ receptor agonist treatment blocked this disruption of the heteromeric complex. Thus, like the Rocheville et al. (2000a) study, this study shows how agonists alone or simultaneous treatment lead to conformational changes in their respective binding pockets that are transmitted to the

heteromeric interface and results in strengthening or disruption of the complex. In this case, the physical interaction is maintained when the $A_1$ and $D_1$ receptor binding pockets are simultaneously activated by agonists allowing the antagonistic intramembrane receptor/receptor interaction to take place, namely the G protein uncoupling with the disappearance of the high-affinity state of the $D_1$ receptor for agonists. One functional meaning of this intramembrane receptor/receptor interaction is therefore uncoupling of the $D_1$ receptor from $G_s$ protein. This is in sharp contrast to the enhanced functional activity of the $SSTR_5/D_2$ receptor oligomers, especially after combined agonist treatment (Rocheville et al., 2000a).

The $A_1/D_1$ heteromeric receptor complex may therefore give the molecular basis for the well documented antagonistic $A_1/D_1$ receptor/receptor interactions found in the neuronal networks of the brain (Ferré et al., 1997; Fuxe et al., 1998, 2002; Franco et al., 2000, 2001). The $A_1/D_1$ receptor heteromerization also appears to have an impact on receptor trafficking (Ginés et al., 2000). Thus, an $A_1$ receptor agonist, after 3 h of exposure, produced a coaggregation of $A_1$ and $D_1$ receptors. On the other hand, a $D_1$ receptor agonist after 3 h of exposure only produced an aggregation of $D_1$ receptor immunoreactivity with a lack of coaggregation in agreement with the ability of the $D_1$ receptor agonist to disrupt $A_1/D_1$ receptor heteromerization (see above). The $D_1$ receptor signaling remained unaffected by the formation of $D_1$ receptor or $A_1/D_1$ receptor clusters, as seen in terms of an unchanged $D_1$ receptor-stimulated cAMP accumulation and thus with no signs of $D_1$ receptor desensitization. In contrast, combined $A_1$ and $D_1$ receptor agonist treatments under the same conditions did not result in the formation of $A_1/D_1$ receptor clusters, but the diffuse $A_1/D_1$ receptor colocalization was maintained. Furthermore, now signs of $D_1$ receptor desensitization developed as seen from reductions in $D_1$ receptor-induced increases of cAMP levels. Thus, essential features of $D_1$ receptor desensitization may be a maintained heteromerization with no $A_1/D_1$ receptor coaggregates formed after prolonged combined exposure to $A_1$ and $D_1$ receptor agonists with no indications of receptor internalization. It seems possible that the $D_1$ receptor desensitization may be mainly caused by a prolonged allosteric change in the $D_1$ receptor brought about by the $A_1/D_1$ receptor/receptor interaction within the heteromeric complex, which could be related to subsequent phosphorylation changes and/or association with $\beta$-arrestin-like molecules (Lefkowitz, 2000; McDonald and Lefkowitz, 2001), leading overall to a reduced $D_1$ receptor/$G_s$ coupling. Thus, it may be suggested that the intramembrane $A_1/D_1$ receptor/receptor interaction in this heteromeric complex is relevant not only for acute antagonism of $D_1$ receptor signaling but also for a persistent long-term antagonism of $D_1$ signaling to the $G_s$ protein. The details of the composition and stoichiometry of the $A_1/D_1$ heteromeric

receptor complex is unknown, and $A_1$ and $D_1$ receptors are known to exist as monomers and homomers (Ciruela et al., 1995; Franco et al., 2000; Lee et al., 2000). It is unknown if heteromers are preferred when $A_1$ and $D_1$ receptors are coexpressed in the same cells.

*h. The Metabotropic Glutamate mGluR$_{1\alpha}$ and Adenosine A$_1$ Heteromeric Receptor Complex.* There is evidence that the group I metabotropic glutamate receptor $mGluR_{1\alpha}$ and adenosine $A_1$ receptors colocalize in certain types of cerebellar neurons and that functional interactions occur between adenosine and glutamate receptors in the brain (Ferré et al., 1999a; Ciruela et al., 2001a,b). Coimmunoprecipitation experiments on soluble extracts from the rat cerebellum synaptosomes have shown that $mGluR_{1\alpha}$ receptors can coimmunoprecipitate with anti-$A_1$ receptor antibodies. Thus, $mGluR_{1\alpha}$ and $A_1$ receptors may exist as heteromers in certain cell populations of the cerebellum (Ciruela et al., 2001a). Subsequent coimmunoprecipitation studies on transiently cotransfected HEK-293 cells showed that $mGluR_{1\alpha}/A_1$ heteromeric receptor complexes exist also in these cells. The receptor subtype specificity was shown by the failure of the COOH-terminal splice variant, the $mGluR_{1\beta}$ to immunoprecipitate with the $A_1$ receptor, indicating the involvement of the COOH-terminal tail in the formation of this heteromeric complex. It is unknown how this heteromeric complex relates to mGluR1 receptor dimer, where the interface modulates the glutamate binding site of the extracellular region of the receptor (Kunishima et al., 2000).

The functional role of this heteromerization was especially studied in the HEK-293 cells. After cotransfection of the $mGluR_{1\alpha}$ and $A_1$ receptors, it was found that quisqualic acid substantially enhanced the increase in $Ca^{2+}$ signaling produced by $A_1$ receptor activation, and the same was true when $A_1$ receptor modulation of $mGluR_{1\alpha}$ receptor function was studied. Thus, it seems as if heteromerization led to the development of synergistic responses in $Ca^{2+}$ signaling upon simultaneous activation of the receptors within the $mGluR_{1\alpha}/A_1$ receptor heteromer. Also in primary cortical cultures, where a high colocalization was observed at dendritic locations, a synergistic interaction was found in terms of a reduction of NMDA-induced neurotoxicity (Ciruela et al., 2001a,b). A reduced hypoxic neuroprotection has been observed in mice lacking $A_1$ receptor (Johansson et al., 2001). It seems possible that the $mGluR_{1\alpha}/A_1$ receptor heteromerization can take place indirectly, since the COOH terminus interacts with specific targeting proteins. Thus, the protein Homer-1a with an enabled VASP homology 1-like domain binds to the COOH-terminal of $mGluR_{1\alpha}$ receptor. Homer-1c can link together proteins with a proline-rich motif (PPSPF), since it binds to this motif (Tu et al., 1999). It is of substantial interest that this or a similar motif is found in both the COOH-terminal part of $mGluR_{1\alpha}$ and in the COOH-terminal part of the $A_1$ receptor. Thus, the Homer can be an important part of

this heteromeric complex as an adapter protein (Xiao et al., 2000; Ciruela et al., 2000). It should also be noticed that the Homer can also link $mGluR_{1\alpha}$ to Shank, since it also contains a similar motif (PPEEF). Shank is a scaffolding multimeric postsynaptic protein that may bring the $mGluR_{1\alpha}$ to the appropriate location on the cell surface and is part of the NMDA receptor-associated PSD-95 complex (Naisbitt et al., 1999; Tu et al., 1999).

*i. The Purinergic $P_{2Y1}$ and Adenosine $A_1$ Heteromeric Receptor Complex.* In 2001, the fascinating finding was made in coimmunoprecipitation experiments on cotransfected HEK-293 T cells that $G_{i/o}$-coupled $P_1$ purinoceptor adenosine $A_1$ receptors can form a heteromeric complex with $G_q$-coupled $P_2$ purinoceptor ATP $P_{2Y1}$ receptors (Yoshioka et al., 2001), showing less than a 5% homology with each other (Fredholm et al., 1994). The COOH-terminal part of $A_1$ receptors was shown not to be involved in this type of heteromerization. In agreement immunofluorescence studies with confocal imaging showed a marked colocalization of the $A_1$ and $P_{2Y1}$ receptors. Furthermore, $A_1$ receptors coimmunoprecipitated with $P_{2Y1}$ receptors indicating that heteromerization between $P_1$ and $P_2$ receptor subtypes could be a rather widespread mechanism for the immediate crosstalk between, e.g., inhibitory $A_1$ receptors and excitatory $P_{2Y1}$ receptors.

A marked change in the signaling was found in the heteromeric complex. Thus, it became possible for the $P_{2Y1}$ receptor agonist $ADP_{\beta}S$ to induce signaling via the $G_{i/o}$ protein coupled to the $A_1$ receptor, an action blocked by pertussis toxin and the $A_1$ receptor antagonist but not by the $P_{2Y1}$ receptor antagonist.

These interesting results seemed to be explained by the development of an ability of the $ADP_{\beta}S$ to reduce $^3$H-labeled $(-)$-$N^6$-phenylisopropyladenosine binding of the heteromeric complex in the high-affinity range. It was therefore suggested that the $A_1$ receptor ligand pocket of the heteromer had markedly changed so as to bind the $P_{2Y1R}$ agonist associated with activation of the $G_{i/o}$ protein. Thus, it was correctly suggested that the heteromeric association produces a $P_{2Y1}$-like $A_1$ receptor. However, it is also possible to propose another molecular mechanism. Thus, there could exist an $A_1/P_{2Y1}$ receptor/receptor interaction at the recognition site level that changes the pharmacology of both the $A_1$ and $P_{2Y1}$ receptor binding pockets. The $P_{2Y1}$ agonist-induced conformational change in the $P_{2Y1}$ receptor binding pocket now not only leads to an activation of $G_q$ proteins but also to a change in the conformation of the $A_1$ receptor, converting it into an agonist state capable of turning on the $G_{i/o}$ protein. Such a conformational change may no longer occur when the $A_1$ receptor binding pocket is occupied by the $A_1$ receptor antagonist locking it into an antagonist state. The $P_{2Y1}$ receptor antagonist may not block the action of the $P_{2Y1}$ receptor agonist since it seems possible that in the heteromeric complex the antagonist used may not have sufficient affinity for the $P_{2Y1}$ receptor binding pocket. The possible existence of the $P_{2Y1}/A_1$ heteromeric receptor complex in the brain could explain the demonstration of theophylline-sensitive $P_{2Y}$ receptors (Mendoza-Fernandez et al., 2000). This $P_{2Y1}/A_1$ heteromeric receptor complex is of substantial interest, since it allows the excitatory ATP receptor $P_{2Y1}$ upon activation to immediately activate in parallel the inhibitory $A_1R$ mechanism. In this way, the excitation and increased energy expenditure brought about by the ATP $P_{2Y1}$ receptor activation begins to be counteracted even at a moment when the extracellular ATP has not been broken down to adenosine, the major ligand for the $A_1$ receptor (Fredholm, 1995a,b; Ferré and Fuxe, 2000; Fredholm et al., 2001).

*j. The Adenosine $A_{2A}$ and Dopamine $D_2$ Heteromeric Receptor Complex.* In 1991, the antagonistic $A_{2A}/D_2$ receptor/receptor interaction was demonstrated in striatal membrane preparations with $A_{2A}$ receptors reducing the affinity of $D_2$ receptors, especially in the high-affinity state, for agonists (Ferré et al., 1991d). This offered a novel mechanism for the reported antagonistic adenosine/dopamine interactions found in the brain (Ferré, 1992, 1997; Fuxe et al., 1993, 1998; Lepiku et al., 1997). The molecular mechanism was proposed to be one of heteromerization of $A_{2A}/D_2$ receptors (Zoli et al., 1993). The same antagonistic intramembrane modulation of $D_2$ receptor recognition mechanisms by $A_{2A}$ receptor activation was observed in different cell lines stably cotransfected with different species and isoforms of $A_{2A}$ and $D_2$ receptors. These were a native $A_{2A}$ receptor/human $D_{2L}$ receptor neuroblastoma cell line (Salim et al., 2000), a dog $A_{2A}$ receptor/human $D_{2L}$ receptor $Ltk^-$ fibroblast cell line (Snaprud et al., 1994; Yang et al., 1995; Dasgupta et al., 1996a), and a human $A_{2A}$ receptor/rat $D_{2S}$ receptor CHO cell line (Kull et al., 1999). This indicated that the same type of intramembrane $A_{2A}/D_2$ receptor/receptor interaction occurs in all cell types and that both $D_{2L}$ and $D_{2S}$ receptors could undergo the same modulation by $A_{2A}$ receptor activation, at least at the recognition site level. The specificity is demonstrated by the failure of $A_1$ receptor agonists to alter the affinity of the $D_2$ receptors (Ferré et al., 1991d). Hillion et al. (2002) have recently reported, based on coimmunoprecipitation experiments, that heteromerization of human $A_{2A}$ and human $D_{2L}$ receptors exists in the basal state in neuroblastoma SH-SY5Y cells stably transfected with $D_{2L}$ receptors and containing native $A_{2A}$ receptors and in fibroblast $Ltk^-$ cells stably transfected with human $D_{2L}$ receptors and transiently transfected with tagged dog $A_{2A}$ receptors. There also exists a high degree of colocalization of $D_2$ and $A_{2A}$ receptors in these cotransfected cells and in primary cultures of rat striatal neurons. The existence of monomers and homomers versus the heteromeric complexes in these cotransfected cells remains to be determined as well as the existence of the simplest heteromeric complex, the $A_{2A}/D_2$ heterodimer. Again, it should be emphasized

that this heteromeric complex exists in the absence of exogenous agonists and the specificity of the $A_{2A}/D_2$ receptor heteromerization is shown by the absence of $A_{2A}/D_1$ receptor coimmunoprecipitation in cells expressing $D_1$ receptors and tagged $A_{2A}$ receptors. One functional meaning of this intramembrane receptor/receptor interaction through heteromerization is then to reduce the affinity of the high-affinity agonist state of $D_2$ receptors. Another meaning is to counteract $D_2$ receptor G protein coupling, since the $A_{2A}$ agonist counteracts the GTP analog-induced disappearance of $D_2$ receptors in the high-affinity state (RH) through a site of action independent of the GTP binding site (Ferré et al., 1993b). Thus, the essence of this $A_{2A}/D_2$ receptor heteromerization may be to convert the $D_2$ receptor into a state of strongly reduced functional activity. In line with this view $A_{2A}$ receptor activation counteracts $D_2$ receptor-induced intracellular $Ca^{2+}$ responses (Salim et al., 2000) and $D_2$ receptor-mediated inhibition of cAMP formation (Kull et al., 1999; Hillion et al., 2002). Based on studies of $D_1/D_2$ receptor chimeras (Kozell et al., 1994; Kozell and Neve, 1997; Torvinen et al., 2001) where the 5th and 6th TM domain plus the IC loop 3 of the $D_2$ receptor has been replaced by the corresponding domain of the $D_1$ receptor, it is likely that these $D_2$ receptor domains are part of the $A_{2A}/D_2$ interface, since the affinity of this $D_1/D_2$ receptor chimera for dopamine can no longer be modulated by the $A_{2A}$ receptor agonist activation (Torvinen et al., 2001). So far it has not been possible to show a reciprocal $A_{2A}/D_2$ receptor affinity regulation by which the $D_2$ receptor upon activation controls the agonist affinity of the $A_{2A}$ receptor.

The $A_{2A}/D_2$ receptor intramembrane receptor/receptor interaction through heteromerization also has an impact on receptor trafficking (Hillion et al., 2002). Thus, coaggregation of $D_2$ and $A_{2A}$ receptors in the cell membrane of neuroblastoma cells could be demonstrated after $A_{2A}$ or $D_2$ receptor agonist treatment for 3 h by means of immunocytochemistry in combination with confocal image analysis of nonpermeabilized cells. The $D_2$ receptor agonist-induced aggregation of $A_{2A}$ receptors was absent in parental neuroblastoma cells, with a very reduced expression of $D_2$ receptors. The increased development of the $A_{2A}/D_2$ receptor coaggregates on the cell membrane after prolonged $A_{2A}$ or $D_2$ agonist treatment was associated with a failure of the $A_{2A}$ receptor agonist to increase cAMP levels. Thus, the $A_{2A}/D_2$ receptor coaggregates that developed were associated with the appearance of both homologous and $D_2$ receptor-mediated heterologous desensitization of $A_{2A}$ receptors.

In contrast, the $D_2$ receptor did not desensitize under these conditions in terms of inhibition of forskolin-induced cAMP accumulation, possibly related to the substantially higher density of $D_2$ receptors, several of which could represent spare receptors. A high degree of colocalization of $A_{2A}$ and $D_2$ receptors was also found in cultured striatal neurons and also here the $A_{2A}$ agonist

or the $D_2$ agonist after a prolonged exposure could induce coaggregates of $A_{2A}/D_2$ receptors.

Evidence for coaggregation followed by cointernalization of $A_{2A}/D_2$ receptors was observed after prolonged cotreatment of the neuroblastoma cells with $A_{2A}$ and $D_2$ receptor agonists. Thus, under these conditions an increase in the uneven distribution of the $A_{2A}/D_2$ receptor immunoreactivity on the membrane was found associated with a marked reduction of the intensity of the immunoreactivity over the $A_{2A}/D_2$ receptor coaggregates. The cointernalization of $A_{2A}/D_2$ receptors could also be directly demonstrated by incubating fluorescent-labeled $D_2$ and $A_{2A}$ receptor antibodies together with $A_{2A}$ and $D_2$ receptor agonists at 4°C for 2 h followed by incubation for 3 h at 37°C, allowing the labeled $A_{2A}/D_2$ receptors to internalize under the influence of the two agonists. Such a synergism with regard to coaggregation and cointernalization of $A_{2A}/D_2$ receptors could not be demonstrated in primary striatal neurons.

It is of substantial interest that in the cAMP accumulation experiments on the neuroblastoma cells, combined agonist treatment was associated with the development of a $D_2$ receptor desensitization as seen from the reduced inhibition by $D_2$ receptor activation of the forskolin-induced cAMP accumulation (Hillion et al., 2002).

Thus, the $A_{2A}/D_2$ receptor heteromerization appears to be involved in the coaggregation, cointernalization, and codesensitization of the $A_{2A}$ and $D_2$ receptors (Hillion et al., 2002). Finally, this intramembrane $A_{2A}/D_2$ receptor/receptor interaction through heteromerization may help understand the cross-tolerance and cross-sensitization found in vivo between dopamine agonists and drugs acting at $A_{2A}$ receptors (Garrett and Holtzman, 1994; Fenu et al., 2000) and also the reduced antiparkinsonian activity and the dyskinesias found after chronic intermittent L-DOPA treatment (Zeng et al., 2000).

*k. The Metabotropic Glutamate mGlu$_5$ and Adenosine A$_{2A}$ Heteromeric Receptor Complex.* In 2002, Ferré et al. were able to demonstrate in cotransfected HEK-293 cells a substantial overlap in the distribution of differentially tagged $A_{2A}$ and the group I metabotropic glutamate receptor mGluR$_5$ receptors. Furthermore, in these transiently cotransfected cells (cDNAs for Flag $A_{2A}$ receptor and hemagglutinin-mGluR$_5$ receptor) coimmunoprecipitation experiments showed that the mGluR$_5$ and $A_{2A}$ receptors formed heteromeric complexes that appeared to be selective since such complexes were not formed between mGluR$_5$ and mGluR$_{1\beta}$. Importantly, $A_{2A}$/mGluR$_5$ heteromeric complexes were also demonstrated in rat striatal membrane preparations with coimmunoprecipitation experiments (Ferré et al., 2002).

These findings are of special interest, since in the striatum the $A_{2A}$ and mGluR$_5$ receptors seem to have a similar distribution pattern in the striatopallidal GABA neurons with a perisynaptic localization to asymmetric postsynaptic, putative glutamatergic synapses (see *Sec-*

tion II.D.). Furthermore, in behavioral studies $A_{2A}$ and mGluR$_5$ receptor agonists synergize in counteracting D$_2$ receptor-induced turning behavior at supersensitive dopamine receptors (Popoli et al., 2001) and, in biochemical studies, both $A_{2A}$ and mGluR$_5$ receptor agonists reduced the affinity of D$_2$ receptor high-affinity agonist binding sites (Ferré et al., 1999a; Rimondini et al., 1999; Popoli et al., 2001). At the moment, it is unknown whether the $A_{2A}$ receptor and the mGluR$_5$ are linked together via direct heteromerization or whether, e.g., the cytosolic Homer proteins are involved that can bind to the COOH-terminal part of mGluR$_5$ and produce their clustering. The Shank proteins having a scaffolding role with multiple protein/protein interaction motives such as proline-rich regions and PDZ domains could also be involved (Milligan and White, 2001), especially since they participate in linking together the mGluR$_5$ with the NMDA receptors (Sheng and Kim, 2000).

The $A_{2A}$/mGluR$_5$ heteromeric receptor complex in the cotransfected HEK-293 cells failed to show synergism in $Ca^{2+}$ mobilization and cAMP accumulation. Nevertheless, a substantial synergism was found after coagonist treatments in terms of MAPK [extracellular signal-regulated kinase 1/2 (ERK 1/2)] and c-fos expression in the cotransfected HEK-293 cells (Ferré et al., 2002). It is presently unknown how signals from the heteromer can bring about this strong synergistic functional interaction that was also observed in the striatum in vivo after combined $A_{2A}$ and mGluR$_5$ agonist treatments (see Section II.D.). Thus, mGluR$_5$ and $A_{2A}$ receptor may mediate glutamate adenosine synergism in case of c-fos expression in the striatum involving the $A_{2A}$/mGluR$_5$ heteromeric receptor complex. There is a distinct possibility that the combined activation of the two receptors of the $A_{2A}$/mGluR$_5$ heteromeric complex may lead to reduced desensitization of the mGluR$_5$ by allowing an increased dephosphorylation to develop thanks to increased activation and/or availability of protein phosphatase 2B at the mGluR$_5$ (Cho and Bashir, 2002; Dale et al., 2002).

It seems likely that the demonstrated synergism in rat striatal expression of c-fos has important functional consequences, since it was matched by a synergism of the mGluR$_5$ receptor agonist CHPG and of the $A_{2A}$ receptor agonist CGS 21680 to counteract phencyclidine-induced motor activity in rats, which is a behavioral response known to be highly dependent on D$_2$ receptor function. It seems possible that the combined activation of the $A_{2A}$ and mGluR$_5$ receptors in the striatum may counteract the well known strong tonic D$_2$ receptor-mediated inhibition of adenylyl cyclase and expression of immediate-early genes in the striatopallidal GABAergic neurons (see Section II.D.). Since immediate-early genes are involved in the connection between short- to long-term adaptive neuronal responses, the $A_{2A}$/mGluR$_5$ heteromeric receptor complex may have a role in striatal plasticity inter alia long-term depression and potentiation as well as in the sensitization to psychostimulants

linked to dopamine-independent c-fos expression (see Section II.D.). Finally, chronic but not acute treatment with a mGluR$_5$ antagonist can reverse a kinetic deficit in a 6-OH-dopamine model of Parkinson's disease (Breysse et al., 2002). This may be related to an altered trafficing of the $A_{2A}$/mGluR$_5$ heteromer, leading to its internalization and/or redistribution allowing a dominance of D$_2$ signaling.

*l. The Bradykinin $B_2$ and Angiotensin $AT_1$ Heteromeric Receptor Complex.* The first indications of the possible existence of a bradykinin/angiotensin II receptor/receptor interaction was obtained by quantitative receptor autoradiography in the nucleus tractus solitarius of the rat brain, a central cardiovascular center (Fior et al., 1993). The findings suggested that in the nucleus tractus solitarius bradykinin B$_2$ receptors were involved in modulating in a differential way the affinity of the high and low affinity binding sites of the angiotensin II (AT$_1$) receptors without effects on the $B_{max}$ values of the AT$_1$ agonist binding sites. Thus, the affinity of the high-affinity agonist state of the AT$_1$ receptors was reduced by bradykinin while bradykinin increased the affinity of the low affinity agonist state using agonist and antagonist radioligands for the AT$_1$ receptor. It was suggested that this receptor/receptor interaction can contribute to the central vasopressor activity of bradykinin by reducing and increasing AT$_1$-mediated transmission at high and low affinity agonist states, considered to be involved in vasodepressor and vasopressor activity, respectively (Fior et al., 1993). However, another interpretation of the results from the competition experiments with an iodinated AT$_1$ receptor antagonist versus angiotensin II (revealing mainly the low affinity agonist component) is that bradykinin reduces the affinity of the AT$_1$ receptor antagonist binding sites, allowing an improved competition by angiotensin II seen as a reduction in the IC$_{50}$ values. Overall it may be considered that the antagonist state of the AT$_1$ receptor can be differentially regulated by B$_2$ receptor activation versus the agonist state. The modulation of the AT$_1$ receptor antagonist binding sites by bradykinin, however, still remains to be determined.

Recently the discovery was made that angiotensin AT$_1$ and bradykinin B$_2$ receptors form heteromers in smooth muscle cells and HEK-293 cells, coexpressing AT$_1$ and B$_2$ receptors (AbdAlla et al., 2000) indicating that this may be the molecular basis for the intramembrane receptor/receptor interactions previously observed between these two receptors.

Immuno-affinity chromatography was performed on proteins from smooth muscle cells and AT$_1$ receptor dimers were coenriched with the anti-B$_2$ receptor antibodies. Since bradykinin and angiotensin II had been cross-linked to the B$_2$ and AT$_1$ receptor antibodies before immuno-affinity chromatography, the results suggested that high-affinity AT$_1$ and B$_2$ receptors form heteromeric complexes on smooth muscle cells. The HEK-293

cells, when expressing only one of the two receptors showed only a monomeric form, but when coexpressing the $AT_1$ and $B_2$ receptors a heteromer was demonstrated, consisting of the $AT_1$ and $B_2$ receptors. The stable $AT_1/B_2$ receptor heteromer could be demonstrated by SDS-PAGE (nonreducing conditions) and was not dependent on agonists but on the density of the two receptors. Thus, it seems likely that intramembrane receptor/receptor interactions reported earlier (Fior et al., 1993) reflect agonist-induced conformational changes in the binding pockets of preformed heteromers leading to alterations in ligand affinity of the other binding pocket. The most impressive finding in the article from AbdAlla et al. (2001) was the increase in the $AT_1$ receptor/G protein coupling in the $AT_1/B_2$ receptor heteromer. This was seen, e.g., by the increased degree of $AT_1$-stimulated redistribution of $G_\alpha$ protein into the cytosol, by the marked increase of angiotensin II-stimulated GTP$\gamma$S binding and the substantial increases in inositol phosphates. An elegant analysis with $B_2$ receptor mutants demonstrated that the $AT_1$ signal increase in the heteromer was dependent on the G protein interface of the $B_2$ receptors but not on the binding of bradykinin to the $B_2$ receptors. The heteromer was, however, formed independently of interference with G protein coupling and with bradykinin binding. Thus, an important functional meaning of the heteromer in this case is the enhancement of $AT_1$ receptor/G protein coupling and thus of $AT_1$ receptor signaling. It has also been shown that the increased presence of the $AT_1/B_2$ receptor heteromer may contribute to the development of angiotensin II hypersensitivity in preeclampsia (AbdAlla et al., 2001).

Still another functional meaning may be a change in receptor trafficking, since the $AT_1/B_2$ receptor heteromer becomes internalized through a dynamin-dependent pathway in contrast to the case when they are expressed alone (dynamin- and clathrin-independent pathway).

*m. Other Heteromeric G Protein-Coupled Receptor Complexes.* It has been reported that also $\beta_2$ adrenergic receptors and $\delta$ opioid or $\kappa$ opioid receptors can undergo heteromerization using coimmunoprecipitation technology (Jordan et al., 2001). Furthermore, protease-activated receptors $PAR_3$ and $PAR_4$ can also form heteromers, where $PAR_3$ is a cofactor for $PAR_4$ activation by thrombin in platelets (Nakanishi-Matsui et al., 2000).

A large number of functional receptor/receptor interactions exists among GPCR in the brain for which evidence of heteromerization is still lacking. This is because there has been no time so far to perform such studies but many are on the way. Based on the evidence for intramembrane receptor/receptor interactions at the recognition level, the following GPCR heteromerizations are postulated: $NK_1/5$-$HT_1$, NT receptor/$D_2$, $CCK_B/D_2$, $A_{2A}/D_3$, Gal receptor/5-$HT_{1A}$, Gal receptor/$\alpha_2$, NPY receptor/$\alpha2$, $AT_1/\alpha_2$, NPY receptor/$AT_1$, $B_2/\alpha_2$, and oxyto-

cin receptor/$\alpha_2$ receptor/receptor heteromerizations (see previous citations in the Introduction, and Härfstrand et al., 1988; Aguirre et al., 1991; Hedlund et al., 1991, 1994; Fior et al., 1994; Yang et al., 1994a, 1996; Fior and Fuxe, 1995; Li et al., 1995a,b; Dasgupta et al., 1996b; Hedlund and Fuxe, 1996; Ferraro et al., 1997; Diaz-Cabiale et al., 2000a–e, 2001; Tanganelli et al., 2001). Finally, muscarinic acetylcholine receptor heterodimerization may also exist (Chiacchio et al., 2000).

## C. Direct Protein/Protein Interactions between G Protein-Coupled Receptors and Multisubunit Ligand-Gated Ion Channels

*1. The GABA$_A$ and Dopamine D$_5$ Heteromeric Receptor Complex.* The direct protein/protein interaction between GABA$_A$ and dopamine D$_5$ receptors was reported in an impressive paper by Liu et al. (2000). A colocalization of GABA$_A$ and D$_5$ receptors was demonstrated in cultured hippocampal neurons by means of immunofluorescence studies in combination with confocal laser microscopy. This was in line with previous work indicating that D$_5$ receptors in hippocampal neurons exist on dendritic shafts and in the axon hillock, regions rich in GABA synapses (Bergson et al., 1995; Nusser et al., 1995).

In Western blots, hippocampal GABA$_A$ receptors could be demonstrated after affinity precipitation with D$_5$ but not D$_1$ receptor COOH-terminal GST fusion proteins. Furthermore, GST fusion proteins with the GABA$_A$ receptor $\gamma_2$ (short) second intracellular loop precipitated the hippocampal D$_5$ but not D$_1$ receptors. These results indicated a physical interaction between the COOH-terminal part of the D$_5$ receptor and the GABA$_A$ receptor $\gamma_2$ subunit, more precisely the second intracellular loop. GABA$_A$/D$_5$ receptor heteromerization was further demonstrated in coimmunoprecipitation experiments. In blot overlay assays, it was shown that the SDS-PAGE separated GST fusion protein of the $\gamma_2$ subunit but no other subunit could directly bind the in vitro translated [$^{35}$S]methionine-labeled D$_5$ COOH-terminal peptide. Likewise, the [$^{35}$S]methionine-labeled second intracellular loop of the $\gamma_2$ subunit could directly bind to the GST-D$_5$ receptor-COOH-terminal fusion protein. It should also be considered that the GST fusion protein of the second intracellular loop of the $\beta_2$ subunit could not bind to the D$_5$ receptor COOH-terminal part in the blot overlay assay in spite of the fact that this part can immunoprecipitate D$_5$ receptors. This interaction may therefore be indirect via associated proteins or involve other parts than the COOH terminus of the D$_5$ receptor. However, this part of the GABA$_A$ receptor may also have a role in the formation of the GABA$_A$/D$_5$ heteromeric receptor complex. It is of interest that there exists a GABA$_A$ receptor-associated protein that can interfere with the ability of the D$_5$ receptor COOH-terminal tail to interact with the $\gamma_2$ intracellular loop (Wang, 2002).

Studies in HEK-293 cells demonstrated that agonist coactivation of $D_5$ and $GABA_A$ receptors (transient coexpression of $GABA\alpha_1$, $\beta_2$, and $\gamma_2$ receptor subunit) was necessary for the coimmunoprecipitation to take place. Thus, agonist-induced changes in the second intracellular loop of the $\gamma$ subunit and in the COOH-terminal part of $D_5$ receptor are essential for the formation of this heteromeric complex. One functional meaning of this heteromerization appears to be to allow mutually inhibitory cross-talk to take place. Thus, in cotransfected HEK-293 cells the $D_5$ but not $D_1$ receptor activation reduced by 30% the $GABA_A$ currents by decreasing the slope of the current-voltage curve. These changes were brought about by a cAMP-independent mechanism and were by means of $D_1/D_5$ receptor chimeras shown to be entirely dependent on the $D_5$ receptor COOH-terminal/$\gamma_2$ interaction in the heteromeric complex formed. Thus, these results indicate that $D_5$ can reduce the synaptic strength over $GABA_A$ receptors via this complex. In agreement with this view, electrophysiological studies in hippocampal slices showed that a $D_1/D_5$ agonist could reduce the amplitude of the $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents independent of protein kinase C (PKC) and PKA. The injection of a GST-encoded $D_5$ COOH-terminal peptide into the recorded neuron prevented this action. This heteromeric complex may therefore be of relevance in vivo, where it may control the synaptic strength of the $GABA_A$ receptor.

Signaling over the $D_5$ receptor was in turn modulated by the $GABA_A$ receptor in an inhibitory way. Thus, in cells coexpressing the two receptors the $GABA_A$ receptor reduced the maximal dopamine activation of the adenylate cyclase by 45%, which was selective for the $D_5$ versus the $D_1$ receptor. This action did not involve changes in the $D_5$ recognition site as shown by absence of changes in dopamine receptor agonist and antagonist affinity nor were any changes in $B_{max}$ values of $D_5$ antagonist binding sites observed. Instead, it seems as if the $GABA_A$ activation of this heteromeric complex reduces the $D_5$ receptor/$G_s$ protein coupling through the $\gamma_2$ intracellular loop 2/$D_5$ receptor COOH-terminal physical interaction. Thus, expression of minigenes encoding the $\gamma_2$ intracellular loop 2 sequences or the $D_5$ receptor COOH-terminal sequences blocked the $GABA_A$ modulation of $D_5$-induced cAMP accumulation. The use of $D_1/D_5$ chimeras gave further evidence for the crucial involvement of the agonist-induced $D_5$ receptor COOH-terminal/$\gamma_2$ complex in the regulation of the G protein coupling of the $D_5$ receptor.

Another functional meaning of this dynamic heteromeric complex may be a role in receptor trafficking (Wang, 2002). Thus, there exist indications that agonist activation of either the $GABA_A$ or the $D_5$ receptor makes possible endocytosis of both receptors and thus cotrafficking (Wang, 2002). It should also be considered that the $\gamma_2$ subunit may be essential for the clustering of the postsynaptic $GABA_A$ receptors. A potential role of this $GABA_A$ receptor/$D_5$ receptor complex in the pathophysiology of schizophrenia was also postulated in view of the fact that alterations in $D_1/D_5$ and $GABA_A$ $\gamma_2$ containing receptors and their functions may exist in the schizophrenic brain (Goldman-Rakic and Selemon, 1997; Okubo et al., 1997; Huntsman et al., 1998; Keverne, 1999).

Very recently Liu and collaborators (personal communication) have obtained evidence that $D_1$ receptors can directly interact with NMDA receptors via protein/protein interaction. Thus, NMDA/$D_1$ heteromerization may have a role in the regulation of glutamate transmission. The demonstration of the $GABA_A/D_5$ and possibly NMDA/$D_1$ receptor complexes are very exciting, since they open up a new way to understand how volume transmission (VT) and wiring transmission (WT) signals can become integrated (Agnati et al., 1987; Zoli et al., 1993; Agnati and Fuxe, 2000). Thus, the $GABA_A$ and NMDA receptors are classical fast synaptic receptors operating via regulation of their ion channels, whereas the GPCR are slow and mainly located extrasynaptically and reached by VT signals in the extracellular space. Heteromerization of ion channel receptors and GPCR offers a new mechanism for the integration of WT and VT and how to control synaptic strength of crucial importance for learning and memory (Abel and Kandel, 1998; Agnati et al., 2002a,b). It may be mentioned that several years ago we obtained indications that $GABA_A$ receptor agonists could modulate the binding characteristics of $D_2$-like receptors in striatal membrane preparations (Pérez de la Mora et al., 1997). Thus, the $GABA_A$ agonist muscimol reduced the affinity of the high-affinity $D_2$ receptor agonist sites as shown in competition experiments with [$^3$H]raclopride versus dopamine. Thus, $GABA_A/D_2$ heteromeric receptor complexes may also exist, since such interactions at the recognition level have been regarded (Zoli et al., 1993) as biochemical indicators of the existence of a heteromeric complex, in this case between $GABA_A$ and $D_2$ receptors. It is also of interest that early on we could report the ability of agonist-activated $\alpha_2$ adrenergic and $D_1$ receptors to substantially modulate [$^3$H]nicotine binding in membrane preparations from the tel-diencephalic regions (Fuxe et al., 1988c, 1989). In view of the above, it seems relevant to test the existence also of heteromeric complexes between nicotinic and $\alpha_2$ adrenergic receptors and between nicotinic and $D_1$ receptors involved in the control of allosteric mechanisms at nicotinic acetylcholine receptors (Changeux and Edelstein, 2001). It is also of substantial interest that cross-inhibition between certain transmitter-gated cation channels (ATP-gated $P_{2X2}$ and $\alpha_3\beta_4$ nicotinic channels) has been shown to exist upon their coactivation (Khakh et al., 2000) probably reflecting heteromerization between these two ion channel receptors when coactivated.

## D. Oligomeric Complexes Containing G Protein-Coupled Receptors and Receptor Tyrosine Kinases

Recently, it has been reported that the epidermal growth factor (EGF) receptors can become associated with growth hormone (GH) receptors and with $\beta_2$ adrenergic receptors upon their stimulation by GH and $\beta_2$ receptor agonists, respectively (Yamauchi et al., 1997; Maudsley et al., 2000). Evidence was provided that their physical association resulted in a transactivation of the EGF receptor. As an example, we will focus on how the $\beta_2$ adrenergic receptor can produce EGF receptor dimerization, tyrosine autophosphorylation, and EGF receptor internalization (Maudsley et al., 2000) leading to MAPK activation. A prerequisite for such a RTK transactivation appears to be the $\beta_2$ agonist-induced formation of a multiprotein complex containing not only the $\beta_2$ adrenergic receptor and the EGF receptor but also $\beta$-arrestin and c-Src, a nonreceptor tyrosine kinase. The coimmunoprecipitation experiments demonstrated the $\beta_2$ adrenergic/EGF heteromeric receptor complex that rapidly formed with a peak within minutes after $\beta_2$ agonist treatment correlated with the $\beta_2$ agonist-induced MAPK activation. This complex could be detected also under basal conditions probably related to a constitutive activity of the $\beta$ adrenergic receptor, since this basal $\beta_2$ adrenergic/EGF receptor complex was markedly reduced by a $\beta_2$ adrenergic receptor inverse agonist. It was shown that Src kinase inhibitors blocked the formation of the $\beta_2$ adrenergic/EGF receptor complex and MAPK activation, indicating a critical role of Src catalytic activity. Furthermore, $\beta$-arrestin recruits c-Src protein to the $\beta_2$ adrenergic receptor (Luttrell et al., 1999) after $\beta$-arrestin has become linked to the $\beta_2$ adrenergic receptor through its agonist-induced phosphorylation via G protein receptor kinase. It was noticed that the transactivation of the EGFR by $\beta_2$ adrenergic receptor was blocked by inhibition of clathrin-mediated endocytosis. It may therefore be that the clathrin-coated pits can represent microdomains for optimizing the signaling among the assembled proteins, leading to the RAS-dependent activation of Raf after the transactivation of the EGF receptor has occurred. As pointed out by Maudsley et al. (2000), these results open up an important mechanism for how GPCRs and also cytokine receptors (Phonphok and Rosenthal, 1991; Quijano et al., 1998) may control trophic signaling, namely through agonist-induced heteromeric complexes with EGF receptors leading to regulation of its transactivation followed by MAPK activation. These studies are excellent examples of how multiprotein complexes form a crucial role also in trophic signaling.

## E. Oligomeric Complexes Containing G Protein-Coupled Receptors and Receptor Activity-Modifying Proteins

### 1. Receptor Activity-Modifying Transmembrane Proteins.

*a. The Calcitonin Receptor Family/RAMP1–3 Heteromeric Complexes.* The calcitonin family peptides such as calcitonin, calcitonin gene-related peptides (CGRP),

adrenomedullin, and amylin act via GPCR. Among others, the calcitonin-receptor-like (CRL) receptor was cloned but could not bind CGRP peptides. An attempt was therefore made to clone the gene for the CGRP receptor by expression cloning (McLatchie et al., 1998). In this study, a single cDNA was finally shown to markedly enhance the responses at an endogenous CGRP receptor in Xenopus oocytes. The cDNA was shown to encode a protein with a single TM domain and an extracellular $NH_2$ terminus (RAMP1). Expression of this protein selectively enhanced CGRP-induced actions in the oocytes. This was the way the RAMP1 was discovered (McLatchie et al., 1998).

Subsequent experiments on cell lines (HEK-293 T cells) demonstrated that coexpression of RAMP1 was necessary for CRL receptor ligand binding (increases in binding of $^{125}I$-$CGRP_1$) and function in terms of cAMP accumulation. The question was how RAMP1 brought about the development of a functional CGRP receptor based on coexpression of CRL receptor and RAMP1. By means of epitope-tagged CRL receptor and RAMP1 in combination with fluorescence-activated cell sorting, it was shown that their coexpression made possible the cell surface expression of both receptors. It was of substantial interest that SDS-PAGE could show cross-linking of $^{125}I$-$CGRP_1$ to two proteins (66,000 and 17,000 bands) from the surface of cotransfected HEK-293 T cells. The two bands seemed to correspond to the native CGRP receptor and RAMP1, respectively, which may form an easily disrupted heteromeric complex according to these findings. Further experiments demonstrated that the coexpression of epitope-tagged RAMP1 and CRL receptor led to the disappearance of a 58-kDa band found with the expression of CRL receptor alone with the appearance of a diffuse 66-kDa band correlating in size with the band cross-linked to $^{125}I$-$CGRP_1$. This additional increase in size by 8 kDa could not be due to an association with the epitope-tagged RAMP1 having a size of $M_r$ 14 kDa. Instead, experiments with endoglycosidases F and H indicated that it was related to a terminal glycosylation of CRL receptor, not found when it was expressed alone and subject only to core glycosylation. The terminal glycosylation of CRL receptor found in the presence of RAMP1 indicates that CRL receptor now can be expressed on the cell surface as a mature glycoprotein, capable of being a CGRP receptor. Nevertheless, it seems likely that also the RAMP1 coexpressed with the terminally glycosylated CRL receptor on the cell surface and capable of becoming cross-linked with $^{125}I$-$CGRP_1$ can contribute to regulation of CRL receptor ligand selectivity and function by physical and/or indirect interactions. It has also recently been observed that multiple amylin receptors can be formed by RAMP interactions with the calcitonin receptor gene product (Christopoulos et al., 1999).

Two other RAMP proteins called RAMP2 and RAMP3 were also discovered (McLatchie et al., 1998), and it was

found that RAMP2 and CRL receptor could generate a receptor for adrenomedullin (ADM). It was found that RAMP2 allowed the transport to and expression of a core-glycosylated CRL receptor (the 58-kDa protein) on the cell surface. This was shown not to be a regular CGRP receptor. It was elegantly demonstrated that oocytes expressing RAMP2 and CRL receptors substantially respond to low concentrations of ADM fragments but only weakly to CGRP. In HEK-293 T cells coexpressing these proteins, specific ADM binding and ADM-mediated increases of cAMP accumulation could be demonstrated. Thus, RAMP2-transported CRL receptor becomes ADM receptors on the cell surface. Based on this work it seems as if the functional meaning of the various RAMPs is severalfold, namely to transport the CRL receptor to the cell surface, to differentially glycosylate the CRL receptor and to interact differentially with the CRL receptor on the cell surface, which may involve heteromeric complexes. All these processes may lead to the development of receptor diversity with markedly different ligand specificities, and CGRP, ADM, and amylin receptor subtypes (see Chen et al., 1997; Perry et al., 1997) can be formed based on the CRL receptor and calcitonin receptor interactions with different types of RAMPs. Thus, CRL receptor and possibly other GPCR can markedly alter its binding pocket by interactions with single TM domain proteins. The role of core and terminal glycosylation versus receptor/RAMP protein interactions resulting in putative heteromeric complexes remain to be determined. When reading about RAMPs one becomes aware of the possibility that many receptors may not function in their own right but mainly as partners in heteromeric complexes and in association with other membrane-associated proteins.

*b. The Dopamine $D_1$ Receptor/Calcyon Heteromeric Complex.* In an attempt to understand how $D_1$ receptors can couple to multiple G proteins, the groups around Goldman-Rakic and Bergson began searching for $D_1$ receptor-interacting proteins with a yeast two-hybrid screen, using the COOH-terminal part of the human $D_1$ receptor as bait (Lezcano et al., 2000). They found a 24-kDa single transmembrane protein, named calcyon, that could interact with the $D_1$ receptor and produce enhancement of $D_1$ receptor-induced $Ca^{2+}$ signaling. The calcyon may therefore be regarded as a RAMP where the interaction is focused on the G protein coupling and not on the binding pocket selectivity as described above for RAMP1–2. The calcyon appears to have an $NH_2$ terminus located extracellularly and a COOH terminus intracellularly located domain like the RAMP1–3 and also contains N-linked oligosaccharides. Immunocytochemistry demonstrated that the $D_1$ receptor and calcyon colocated in the same population of pyramidal cells of the cerebral cortex and in a subpopulation of $D_1$ receptor-containing striatal neurons. It is of interest that both calcyon and $D_1$ receptors were located

perisynaptically in dendritic spines at a postsynaptic location.

Coimmunoprecipitation experiments indicated that $D_1$ receptor and calcyon formed a heteromeric complex in HEK-293 cells. Furthermore, using a GST fusion protein with the $D_1$ receptor bait sequence ($GSTD_1$) and a bacterial fusion protein with the COOH-terminal part of calcyon (S-calcyon), it could be shown that $GSTD_1$ became bound to S-calcyon. Thus, a direct COOH-terminal interaction may be involved in the formation of this calcyon/$D_1$ receptor heteromeric complex, where the $D_1$ receptor COOH-terminal sequences 421–435 appear crucial since this peptide prevented the binding.

The formation of the calcyon/$D_1$ receptor heteromeric complex resulted in a marked change in $D_1$ receptor signaling in HEK-293 cells. After priming by activation of ATP $P_{2Y}$ receptors but not otherwise, the $D_1$ receptor agonist SKF 81297 produced a rapid increase in $Ca^{2+}$ signaling dependent on release from intracellular $Ca^{2+}$ stores provided that transfection with calcyon had been performed. This $Ca^{2+}$ response was similar in size to that produced by the $P_{2Y}$ receptor linked to Gq/11.

These results can be explained by assuming that the $P_{2Y}$ activation can increase the coupling of the $D_1$ receptor to the Gq/11 protein, an increase that may be further strengthened by the formation of the $D_1$ receptor/calcyon complex. In contrast, the $D_1$ receptor signaling over the $G_s$ to adenylyl cyclase leading to increases of cAMP accumulation was unaltered by the formation of this complex. Thus, the dual signaling of the $D_1$ receptor with involvement also of $Ca^{2+}$ signaling via Gq/11 becomes more pronounced by this heteromeric complex with calcyon.

Formation of this complex appeared to be inhibited by expression of $D_1^{421-435}$ in the cells, which reduced the $D_1$ receptor-stimulated $Ca^{2+}$ responses probably by competing with the $D_1$ receptor for calcyon. The heteromeric complex formation is increased by the $D_1$ receptor activation and reduced by a PKC inhibitor. In fact, the COOH-terminal part of the calcyon can become phosphorylated by PKC and may bind phosphoinositol-4,5-biphosphate. These observations give an increased understanding to the importance of the direct interaction between the COOH-terminal parts of $D_1$ receptor and calcyon for the increased Gq/11 protein coupling of the $D_1$ receptor with activation of phospholipase C (PLC) and formation of inositol 1,4,5-trisphosphate ($IP_3$) (see *Section II.D.* for details of a Gq-mediated signaling).

It is of substantial interest that stimulation of endogenous muscarinic Gq/11-coupled $M_1$ receptors, like ATP $P_{2Y}$ activation, prior to $D_1$ receptor activation also made possible a strong $D_1$ receptor-induced increase in $Ca^{2+}$ signaling by the heteromeric $D_1$ receptor/calcyon complex. It seems possible that the mechanism underlying the primary actions of ATP and $M_1$ receptor activation can be severalfold with the end result being an increased Gq/11 coupling of the $D_1$ receptor. Thus, the indication of

multiple receptor interactions with formation of high order heteromeric complexes containing ATP and $D_1$ receptors and calcyon or $M_1$ and $D_1$ receptors and calcyon may be considered leading to the increase of $D_1$ receptor coupling to the Gq/11 protein. The activation of intracellular phosphorylation cascades involving, e.g., PKC can also be involved with phosphorylation of the COOH-terminal calcyon, especially since the priming action have so far only been observed with GPCR coupled to Gq/11.

The pioneering work of Goldman-Rakic has demonstrated the prominent role of the $D_1$ receptor in the cerebral cortex in the modulation of glutamate receptor signaling during working memory operation (Williams and Goldman-Rakic, 1995). This work will therefore have relevance for understanding cortical plasticity. It is also inspiring that $M_1$ receptors, like $D_1$ receptors, are located on dendritic spines of pyramidal nerve cells (Mrzljak et al., 1993). Thus, $M_1/D_1$ receptor/receptor interaction via possible heteromeric complexes may therefore exist in dendritic spines and have a functional relevance (Wang and McGinty, 1997).

*2. Receptor Activity-Modifying Cytosolic Proteins.*

*a. The Adenosine $A_1$ Receptor and Adenosine Deaminase Heteromeric Complex.* Franco and collaborators (Franco et al., 1997, 1999, 2000) have obtained evidence that adenosine deaminase (ADA) can form a heteromeric complex with adenosine $A_1$ receptors. ADA is an enzyme capable of converting adenosine in inosine. ADA is also a multifunctional protein appearing on the cell surface anchored to different proteins (Lluis et al., 1998; Mirabet et al., 1999, Herrera et al., 2001). It can therefore act enzymatically but also extraenzymatically (Franco et al., 1997, 1998) as in the case of the formation of ADA/$A_1$ receptor complexes. Formation of ADA/$A_1$ receptor complexes was demonstrated in experiments involving confocal laser microscopy, coimmunoprecipitation, and affinity chromatography. Thus, ADA and $A_1$ receptors coimmunoprecipitated and $A_1$ receptors were retained in a matrix of ADA-Sepharose. Finally $A_1$ receptors colocalized with ADA on cell membranes, including cell surface cortical neurons (primary culture; Ruiz et al., 2000). The binding of ADA to the $A_1$ receptors appears to be essential for the high-affinity agonist binding of $A_1$ receptors, giving a functional role of this physical interaction in $A_1$ receptor-mediated transmission (Ciruela et al., 1996; Saura et al., 1996, 1998). Thus, ADA has a role not only as a degradative ectoenzyme but also as an $A_1$ receptor activity-modulating protein. It therefore became of interest to study a possible role of ADA in the $A_1/D_1$ heteromeric receptor complex. In a recent study (Torvinen et al., 2002), using the same $A_1/D_1$ receptor-cotransfected fibroblast cell line as described above, evidence was obtained that in nonpermeabilized $A_1/D_1$ receptor-cotransfected cells, but not in cells only transfected with $D_1$ receptor, ADA exists on the plasma membrane. These results indicated that ADA can be targeted

to the membrane by $A_1$ receptors but not by $D_1$ receptors. Futhermore, $A_1$ receptor agonist (R-PIA, 100 $\mu$M, 3 h) preincubation resulted in coaggregations of both $A_1$ receptors and ADA and $D_1$ receptors and ADA in the $A_1/D_1$ receptor-cotransfected fibroblast cells. These results suggested that after $A_1$ receptor agonist treatment with maintained $A_1/D_1$ heteromerization coaggregates are formed that contain high-order molecular structures (Torvinen et al., 2002) involving ADA/$A_1$ receptor/$D_1$ receptor heteromeric complexes and other interacting proteins that have a special functional role, especially in receptor trafficking. However, ADA does not seem to be linked directly to $D_1$ receptors. In line with this view, ADA/$D_1$ receptor aggregates are no longer present after $D_1$ receptor agonist pretreatment (SKF 38593, 10 $\mu$M, 3 h), disrupting the $A_1/D_1$ receptor heteromerization leading to aggregated $D_1$ receptor alone, while ADA/$A_1$ receptor immunoreactivity remain diffusely colocalized (Torvinen et al., 2002). The impact of the ADA/$A_1$ receptor complex on $D_1$ receptor signaling was demonstrated by the blockade of the $A_1$ agonist-induced uncoupling of the $D_1$ receptor by the irreversible blockade of ADA function using deoxycoformycin. This counteraction was unrelated to the rise of endogenous adenosine levels (Torvinen et al., 2002). Thus, ADA is part of the $A_1/D_1$ heteromeric receptor complex but directly linked only to the $A_1$ receptor, where it makes possible the high-affinity state of the $A_1$ receptor for agonists, allowing it to modulate the operation of the $D_1$ receptor.

*b. The Adenosine $A_1$ Receptor and hsc73 Heteromeric Complex.* Sarrió et al. (2000) demonstrated that adenosine $A_1$ receptors interact with a protein of the family of heat shock proteins. By affinity chromatography the heat shock cognate protein hsc73 was identified as a cytosolic component able to interact with the third intracellular loop of the receptor. As demonstrated by surface plasmon resonance, purified $A_1$ receptors interact specifically with hsc73 with a dissociation constant in the nanomolar range ($0.5 \pm 0.1$ nM). The hsc73/$A_1$ receptor interaction leads to a marked reduction in the affinity of $A_1$ receptor agonist ligands, a reduction of $A_1$ receptor antagonist binding, and prevents activation of G proteins, as deduced from [$^{35}$S]GTP$\gamma$S binding assays. Interestingly, this effect on $A_1$ receptor agonist binding was stronger than that exerted by guanine nucleotide analogs, which uncouple receptors from G proteins, and was completely prevented by ADA, which interacts with the extracellular domains of $A_1$ receptors (see above). As assessed by immunoprecipitation, a high percentage of $A_1$ receptors in cell lysates are coupled to hsc73.

Members of the hsp70 family interact with a number of cellular proteins. Due to the molecular chaperone function of hsp70 proteins, they appear capable of recognizing "non-native" forms of proteins. This is not the case for $A_1$ receptors for some reasons. One is due to the fact that solubilized $A_1$ receptors are very sensitive to the composition of the medium, which affects ligand

binding to the soluble molecule. Thus, a precise combination of detergent and salts is required to achieve a high recovery of binding sites in solubilized preparations of $A_1$ receptors. The strong effect of nanomolar concentrations of hsc73 upon ligand binding to purified soluble $A_1$ receptors is evidence for a specific interaction between hsc73 and functional $A_1$ receptors. The specificity of the interaction has also been demonstrated in primary cultures of neurons where other GPCR ($A_{2A}$ receptors, $A_{2B}$ receptors, or metabotropic glutamate mGluR$_4$ receptors) do not colocalize with hsc73.

On the other hand, colocalization between $A_1$ receptors and hsc73 is not restricted to a specific zone of the cell, even in cells naturally expressing the proteins, and this is evidence that the interaction occurs with completely folded functional receptors. Apart from the regulatory role of the interaction in ligand binding, there are data supporting the idea that hsc73 is relevant for the trafficking of the $A_1$ receptors. As a matter of fact, colocalization between hsc73 and $A_1$ receptors was detected in specific regions of rat cerebellum and in the nerve cell bodies of cortical neurons but not in dendrites or synapses. Moreover, it seems that agonist-induced receptor internalization leads to the endocytosis of $A_1$ receptors by two qualitatively different vesicle types, one in which $A_1$ receptors and hsc73 colocalize and another in which hsc73 is absent. These results open the interesting possiblity that the signaling and trafficking of GPCR may be regulated by heat shock proteins.

The novel findings presented by Sarrió et al. (2000) suggest a specific role for hsp70 proteins in regulating the activation and operation of $A_1$ receptors. Although a relevant role for chaperones in signaling by steroid hormone receptors has already been demonstrated (Bohen et al., 1995; Caplan et al., 1995), our results are the first evidence suggesting a control by hsc73 of signaling via plasma membrane GPCR. It should also be noted that this member of the GPCR can be regulated differently by a protein interacting with extracellular domains of the receptor (ADA, see above) and by a cytosolic protein interacting with the third intracellular loop of the receptor (hsc73).

## II. On the Functional Implications of Receptor/Receptor Interactions

### A. The Context of the Present Discussion

The existence of homodimers, heterodimers, homo-oligomers, and hetero-oligomers of GPCR provides the structural framework to explain the function of GPCR in a variety of systems. From a biochemical point of view, the formation of GPCR homo- and heteromers explains some of the data on ligand binding and of cross-talk that have been reported for many years in the literature and that were interpreted in an erroneous or incomplete way. The knowledge of the molecular mechanisms underlying receptor function introduces, however, complexities derived from the fact that different conforma-

tions of a single receptor may arise and that receptor molecules with different conformation and from different receptors can interact to give rise to multiple oligomeric structures in specific membrane microdomains. The role of structural diversity in GPCR function will be the topic covered first in this section (Gutkind et al., 1998; Bockaert and Pin, 1999; Gether, 2000; Heuss and Gerber, 2000; Lefkowitz, 2000).

On the other hand, it is important to give an example of how heteromerization of the receptors can improve our understanding of how signals are integrated in a given system. Since substantial indirect evidence for receptor/receptor interactions has been provided, studying receptors for neurotransmitters and recent evidence for heteromeric receptor/receptor interactions has been given in the central nervous system, a complete and comprehensive account of functional implications of certain receptor/receptor interactions occurring in the basal ganglia is provided. Thus, the interactions between adenosine, dopamine, and glutamate metabotropic receptors in the GABAergic striatopallidal neurons will be covered in full. It should be noted that the heteromerization concept gives new therapeutic directions for treatment of diseases involving inter alia this brain region, such as Parkinson's disease, schizophrenia, and drug addiction.

### B. Structural Basis of Receptor Function

*1. Conformational Diversity.* It seems likely that the receptor can assume not only two conformations related to two functional states: active (R*) versus inactive (R) receptor, but rather several slightly different conformations. In fact, proteins can assume a large number of slightly different structures each of which with potentially different biochemical characteristics.

The pharmacology of GPCR has led to the frequent finding of negative cooperativity in agonist binding. In fact, the work of Limbird et al. (1975) and Limbird and Lefkowitz (1976) gave clear evidence for negative cooperativity for binding of adrenergic agonists to $\beta$ adrenergic receptors, which was further demonstrated for a variety of GPCR. In the case of catalytic proteins a negative cooperativity could be explained only in multimeric enzymes and assuming that there was intercommunication between the enzyme subunits. In a scenario where GPCRs were assumed to be present as monomers in the membranes, negative cooperativity was difficult to explain. One possible explanation for this apparent negative cooperativity was the assumption that a given receptor can exist in two conformational states with different affinity for the agonist. It is frequently assumed that the high-affinity form is due to the conformation of the receptor/G protein complex whereas the low affinity form is due to the conformation of the receptor uncoupled from the G protein. These coupled and uncoupled receptor states would correspond to the functionally active (R*) and inactive forms (R). The view that

some of the GPCRs displaying negative cooperativity existed in membranes as two totally independent conformations had to change by the finding that the affinity constant deduced from equilibrium binding or kinetic binding experiments were different (Casadó et al., 1991). This should not be the case for two independent affinity states.

The existence of receptor dimers would lead to an explanation of the kinetic data on agonist binding assuming that there is negative cooperativity among the two interacting receptor monomers. The truth may be not as simple as that. The occurrence of clustering clearly suggests that G protein-coupled receptors form high order molecular structures, in which multimers of the receptors and probably other interacting proteins form functional complexes (Fuxe and Agnati, 1987). Therefore, negative cooperativity has to be explained taking into account the variety of conformations in which a given GPCR can exist in the cell membrane. There is a model for GPCR operation that takes into account that different GPCR molecules can display different conformations at a given time and that agonist binding can change this conformational pattern. The *cluster-arranged cooperative model*, which accounts for the kinetics of ligand binding to adenosine $A_1$ receptors (Franco et al., 1996), shows that high- and low-affinity sites are a consequence of the negative cooperativity of agonist binding and may not be related to the content of free and G protein-coupled species. Conceptually this model takes advantage of the fact that GPCRs are not isolated proteins but linked with other components of the membrane. This intramembrane intercommunication is the basis of the observed negative cooperativity and can even participate in the multiple processes involved in ligand-induced desensitization. Assuming that the GPCR communicate with each other in the membrane, the idea of the model is that agonist binding decreases the affinity of receptors that are not yet interacting with the ligand. The molecular mechanisms of this effect are multiple and probably vary from GPCR to GPCR. The validity of the model was proven with $A_1$ adenosine receptors, which cluster after agonist binding (Franco et al., 1996). For this receptor, it is likely that clustering is one of the factors affecting the conformation of the receptor in such a way that it decreases the affinity for the agonist. This is also the reason that the model was named as *cluster-arranged cooperative model*. The model considers that the cluster may be formed by molecules other than the $A_1$ receptor itself, and therefore, it is open to heteromeric and multimeric interaction involving two or more proteins and even membrane lipids (Franco et al., 1996). This broad conformational spectrum that is possible for GPCR confers functional plasticity of GPCR since the conformational pattern and thus the energy landscape in response to a variety of effectors, chemical and also physical stimuli will indeed vary. These physicochemical-induced influences on the GPCR energy landscape is homologous to what happens in computational processes (Frauenfelder et al., 1991). Therefore, GPCRs are capable of detecting the specific features of the cellular microenvironments in which they are embedded to assume a certain preferential conformation, which in turn affects function (Fig. 1). Thus, the following can be proposed (Agnati et al., 2002a,b):

- GCPR conformation at a given circumstance is the transient result of the chemical-physical factors acting upon the receptor and coming simultaneously from the extracellular space (Ciruela et al., 1996; Saura et al., 1996, 1998), the membrane (Casadó et al., 1990, 1991, Ginés et al., 2001), and the intracellular space (Sarrió et al., 2000).
- Ligand induces conformational changes and conformational changes in the receptor would affect ligand binding and, therefore, signaling (Franco et al., 1996).
- The chemical-physical inputs can lead to a conformation that weakens the intramolecular stabilizing interactions favoring constitutive ligand-independent activation of the receptor [see the so-called "protonation hypothesis" for GPCR conformation (Gouldson et al., 2000)].
- The GPCR conformation may affect signaling; that is, one GPCR conformation may favor the activation by the receptor of certain particular decoding pathways (Kenakin, 1995, 1997). It should be noted that signaling is also dependent on the G protein interacting with the GPCR, but again, it is reasonable to
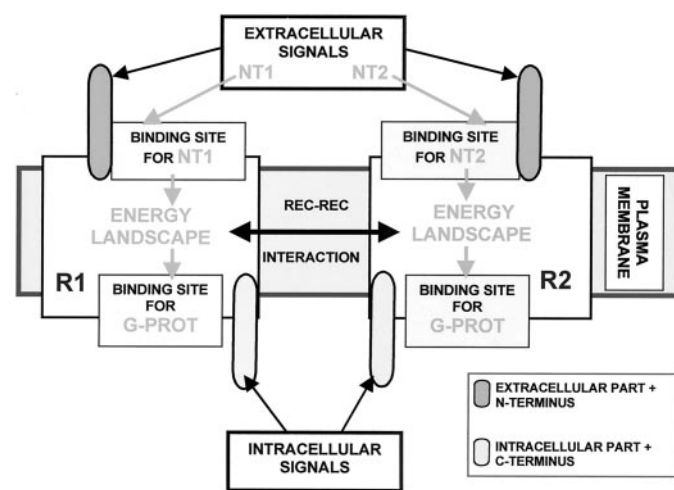
FIG. 1. Schematic representation of the GPCR as a sensor of the extracellular, intramembrane, and intracellular microenvironments. Thus, at least three aspects should be considered: first, the steric conformation of a protein is deeply affected by the microenvironment in which it is embedded, and the GPCR spans three different microenvironments (extracellular, intramembrane, and intracellular microenvironments); second, the GPCR has two long tails ($NH_2$ terminus, COOH terminus) that can very easily change shape as a result, e.g., of the ionic force of their respective microenvironment; third, changes in GPCR conformation can deeply affect both its ligand binding characteristics and its binding to the G proteins. NT1, neurotransmitter 1; NT2, neurotransmitter 2; R1, receptor 1; R2, receptor 2.

assume that a given G protein interacts preferentially with a given conformation of the receptor (Cordeaux et al., 2000).

- The GPCR conformation may affect receptor turnover, and thus signaling and receptor trafficking (Ginés et al., 2000; Hillion et al., 2002).
- As a result of the above, the conformational pattern and thus the energy landscape is the key for the output in terms of function.

*2. Oligomeric Diversity: The Receptor Mosaic* After the reports indicating the existence of heterodimers and heteromers of GPCR, the present data on receptor/receptor interactions cannot distinguish between domain-swapped dimers/heteromers and contact dimers/heteromers (Gouldson et al., 2000; Schulz et al., 2000). It is our opinion that very likely both types of interactions (domain-swapping and domain contact) can occur allowing the formation of high order hetero-oligomers. The prevalence of one of the two ways may depend on the receptor type and on the chemical-physical environments in which the interacting receptors are embedded that affect GPCR conformations (see above).

Recent papers have carefully discussed the mechanistic aspects of receptor/receptor interactions (Gouldson et al., 2000). Based on this work, it is possible to distinguish two pseudoindependent units in the GPCR; thus the NH₂-terminal and helices 1 through 5 constitute the A-GPCR domain, whereas helices 6 and 7 through to the COOH terminus constitute the B-GPCR domain. The two A and B domains are connected by a hinge loop (ICL3) that is, frequently, the longest loop in GPCR and therefore very well suited to allow reciprocal movements of the two A and B-GPCR domains. It has been suggested that helices 5 and 6 form the dimerization interface and the 5 and 6 domain-swapped dimer may be the active (R*) form of the receptor that interacts with the G protein. Examination of various structures of adrenergic receptors by means of molecular dynamics suggests that the role of the agonist may be that of stabilizing the 5 and 6 dimer through conformational changes in helices 5 and 6. One problem is the dilemma whether agonist promotes dimerization or dimers are already preformed. It has been suggested that dimers are preformed and merely rearranged in the presence of the agonist (Gouldson et al., 2000). Although this can happen for some receptors (Ginés et al., 2000; Hillion et al., 2002), current evidence indicates that agonists affect markedly the oligomerization state of some GPCR (see Table 1 and Franco et al., 1996). It is important to underline that functional sites have been identified on the external face of helix 2, which could be involved in the formation of heterodimers and thus in the formation of high order hetero-oligomers as well as in heterodimerization processes with other proteins.

This view is in agreement with the receptor mosaic hypothesis suggested in 1982 (Agnati et al., 1982, 1990, 2002, 2003; Fuxe et al., 1983a; Zoli et al., 1996), with the cluster arranged cooperative model and with the fact that quite often agonists lead to clustering of GPCR cell surface receptors (Franco et al., 1996). Taking into account that oligomeric complexes are likely composed of ordered conglomerates of a number of different membrane, extracellular and intracellular proteins, such clusters were viewed as computational units having an important role in the information handling by the cell and were defined by Agnati et al. (1982) as a "receptor mosaic" or, more generally, a "protein mosaic" that may contain also other plasma membrane-associated proteins (Fuxe and Agnati, 1985; Agnati et al., 2002, 2003).

It is reasonable to think that the composition, geometry, and characteristics of this protein mosaic will depend on the above-mentioned physical-chemical factors, and in turn, the characteristics of the mosaic will target specific patterns of signaling pathways. Therefore, activation of one receptor would lead to different cell responses depending upon the nature of the mosaic where the receptor is located. It is evident that different cells, or the same cell in different conditions (activation, mitosis, etc.) could lead to different signaling scenarios for a given receptor since the composition and function of the mosaic will depend on the pattern of trafficking and energy landscape (conformational state) of the proteins forming the mosaic (Agnati et al., 2003).

Some basic features of a receptor mosaic with functional consequences are described as follows (Agnati et al., 2002):

- A receptor cluster works as a receptor mosaic if and only if the cluster of proteins (heteromeric receptor complex involving GPCR and/or other proteins as ion channel receptors, tyrosine kinase receptors or receptor activity-modulating proteins) is such that activation of a receptor modulates the biochemical/functional features of at least another receptor of the cluster, i.e., if and only if receptor/receptor interactions are in operation in the cluster of molecules, where adapter protein may play an important role in stabilizing the receptor mosaics.
- The fluctuation of each receptor (of the receptor mosaic) among its possible conformational states is conditioned by the conformations of the other receptors in the receptor mosaic (receptor/receptor interactions). Hence, each receptor will respond to its ligand in a way that depends on its conformation and thus on its interactions with the other receptors of the macromolecular complex.
- The receptor mosaic is connected with effector proteins (e.g., G proteins, ion channels) and works as an integrative unit.

It should be noted that proteins can work as modules capable of building up supra molecular complexes (Pawson, 1995; Pawson and Scott, 1997; Pawson et al., 2001).

This can be used for a variety of molecular events, for instance, to "hide" functional groups of a protein or to have emergent complex functions performed by protein aggregates. On the other hand, protein/protein association is, inter alia, strongly regulated by protein phosphorylation (Huganir and Greengard, 1987; Greengard et al., 1998, 1999; Hunter, 2000). Examples of protein/protein interactions are the receptor/receptor interactions, the assembly of $\alpha\beta\gamma$ subunits of the G proteins, and those interactions occurring along the MAP kinase pathways (Impey et al., 1999). Some of the possible interacting proteins can work indirectly as "scaffolds" (Pawson, 1995; Luttrell et al., 1999, 2001; Kohout et al., 2001; Miller and Lefkowitz, 2001). These proteins do not have the capability to transfer or elaborate information, but provide a scaffold along which other proteins transfer and/or elaborate information in an ordered and efficient fashion. For example, they provide a scaffold along which a series of enzymes process their substrates in a well defined sequence and with an efficiency, specificity, and rate that is clearly not possible in a freely diffusing system. These scaffolding proteins have indeed a relevant role that must be taken into account to understand how recognition occurs and signaling is transmitted. They may even be involved in processes such as those related to memory in the central nervous system.

Aspects on receptor activation should be revised in the light of the receptor plasticity and especially in light of receptor/receptor interactions. The different models of receptor activation that have been proposed (Kenakin, 1995, 1997) such as the two-state model versus the multistate model and the conformation selection versus conformation induction of the receptor binding site are still useful. The *cluster arranged cooperative model* (see above) has given a new perspective on the ligand binding and activation of GPCR (Franco et al., 1996). However, on the basis of the recent evidence on GPCR plasticity and on the suspected complexity of receptor mosaics, a new more global model should be devised. This model should take into account that the receptor recognizes and decodes the neurotransmitters or other physical-chemical signals according to the inputs that it receives from the other proteins of the membrane (in particular the other receptors of the cluster) and from the extracellular and intracellular signals impinging on it. On the other hand, the receptor mosaic concept can help in understanding better at a molecular level how complex integrative tasks performed by neurons (such as memory, see below) can take advantage of molecular circuits located at the plasma membrane level.

*3. The Role of the Receptor Mosaic in Learning and Memory.* Grant and Husi (2001) have elegantly described the synaptic multiprotein complexes associated with the NMDA receptors and the PSD-95 (postsynaptic density protein 95) and how they can process information and encode memories (Migaud et al., 1998). In recent articles (Agnati et al., 2002a,b) building on earlier

articles (Agnati et al., 1982; Fuxe et al., 1983a), we have postulated the existence of three-dimensional molecular circuits built up of intramembrane receptor mosaics, ion channels, and G proteins linked to cytosolic protein networks of protein kinases, protein phosphatases, scaffold, and anchoring proteins (Weng et al., 1999). These three-dimensional molecular circuits can store information in nerve cells, where engram formation may depend on the resetting of receptor mosaics (higher order hetero-oligomeric complexes). Transient stabilization of the receptor mosaic is postulated to result in short-term memory associated with an appropriate change of synaptic weight. Long-term memory, i.e., engram consolidation, may according to our hypothesis be caused by the ability of the molecular circuits involved to form intracellular signals translocating to the nucleus and activating a large number of transcriptional factors leading also to induction of various immediate early genes. In this way inter alia a number of postulated unique adapter proteins can be formed that will stabilize the receptor mosaic and lead to the consolidation of the receptor mosaic so that it becomes a long-lived heteromeric receptor complex (Agnati et al., 2002a,b). It will then, when again activated, induce ion channel activity and protein kinase and phosphatase cascades in its molecular circuit that will develop the synaptic weight at the time of the perception of the event to be remembered, and the retrieval of the engram can take place, since the appropriate pattern of firing rate in the nerve cell can occur. It must be underlined that this theory on the molecular basis of learning and memory is in agreement with the Hebbian rule stating that learning is associated with simultaneous changes in pre- and postsynaptic activity. According to our theory, the change of presynaptic activity will cause the specific change of postsynaptic activity by reorganizing the receptor mosaics of the postsynaptic membrane, leading to novel or altered molecular circuits that upon integration with other available three-dimensional molecular circuits in the nerve cell will produce a change of the firing rate pattern that is linked to the change in presynaptic activity.

The development of long-lived heteromeric receptor complexes of high order may, in the nerve cell membrane, therefore be the molecular basis for learning, memory, and retrieval (Hobb, 1949). Here the important work on cooperativity in biological membranes and of clustered receptors must also be mentioned (Changeux et al., 1967; Duke and Bray, 1999) and is in line with this hypothesis.

### C. Communication Processes in the Cell

The concept to be introduced here is derived in part from studies on the communication processes at the level of neural networks (Agnati and Fuxe, 2000). Thus, the communication within a compartment and between compartments in the cell may occur via WT as well as via VT. In the first instance, there is the channeling of

the information along some sort of physical delimited pathway (the information channel), e.g., a set of proteins interconnected or working along scaffold proteins (Weng et al., 1999). In the second instance, there is the diffusion of signals in the cytoplasm (possibly along preferential diffusion pathways) to reach the proper target, where the signal is recognized (bound) by the target molecule, and the message is decoded (Fig. 2). Although VT is very likely important both for communication between different compartments and within compartments, WT is mainly used for communication inside a compartment. It should also be noticed that to get WT, besides molecular scaffolds, the cell can use other specific nonleaking routes such as the directional transport of signaling proteins via filament-bound motor proteins or via the vesicular transport.

The modular nature of the signaling pathways and the process of protein/protein interactions (Edwards and Scott 2000; Fraser et al., 2000, Grant and Blackstock, 2001; Marinissen and Gurkind, 2001; Pawson et al., 2001) lead to "wired molecular circuits" that can be traced from the plasma membrane compartment, through the cytoplasm, toward the nuclear compartment. Receptor mosaics represent not simple inputs to these "vertical molecular circuits" but rather "horizontal molecular circuits" capable of integrating different signals already at the plasma membrane level (Fig. 3). In fact, due to the spatial/temporal contiguity of the transductional processes not a single (elementary) response but a complex activation of several intracellular signaling pathways occurs. These pathways cross-talk, in a controlled fashion, at several levels. This leads to biochemical/ionic responses that are the result of integrations at several crucial levels ("nodal points" of the signaling pathways) and thus to a "syndromic response" by the cell (Figs. 2 and 4). It should be noticed that, according to this view, the receptor mosaic has to be considered as a nodal point of signaling pathways at the membrane level.

As pointed out above, intracellular signaling pathways can use both WT and VT. In addition, cross-talks among signaling pathways are made possible by both WT and VT. In fact, there are nodal points where VT messages can enter a "wired signaling pathway" as well as there are nodal points where several wired signaling pathways converge. Via this cross-talk among intracel-
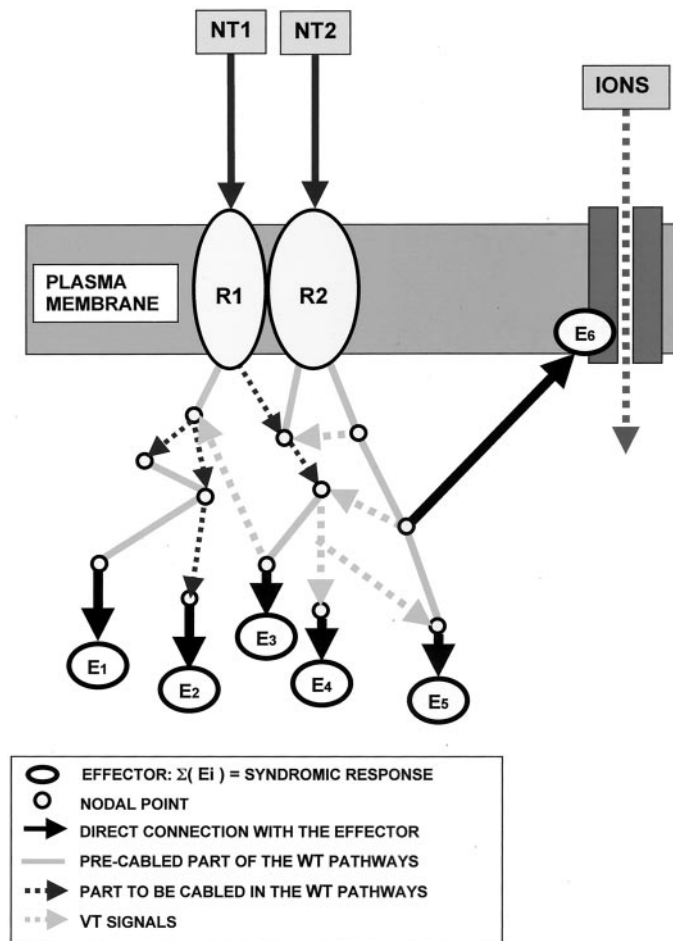
FIG. 2. Two modes for the communication process inside of a cell are illustrated, the WT and the VT. The WT is the transmission of signals along a nonleaking channel (e.g., assembled protein modules that work as a "wire"), whereas VT is the transmission of signals along leaking channels (i.e., free diffusion of signals in the cytoplasm medium). Sites (nodal points) where integration of WT and VT signals can take place are indicated. Thus, it may be underlined that the pattern according to which signaling pathways converge toward nodal points should be identified since each of these potentially different arrangements represents an important feature of the handling of information by the cell (see also Fig. 4). How a "syndromic response" is generated is also shown.
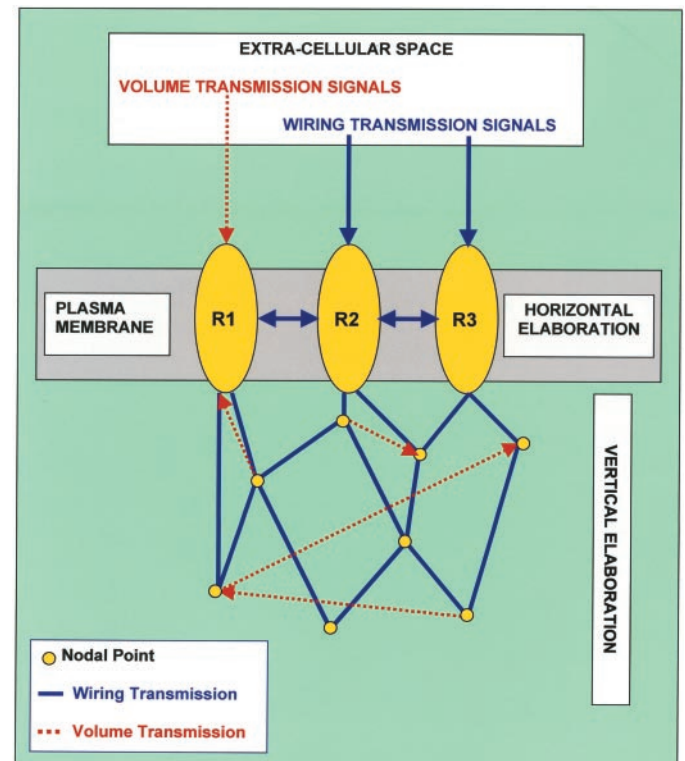


FIG. 3. Signaling pathways inside the cell and how a preferential horizontal (i.e., in the plane of the membrane) versus a vertical (i.e., through the cytoplasm toward the nuclear compartment) elaboration can be distinguished. For further details, see text.
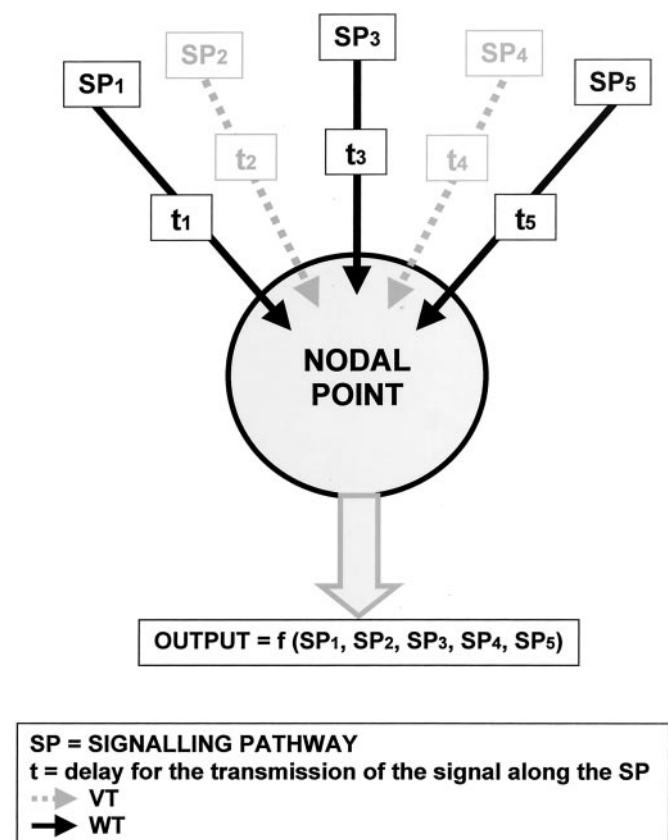
Fig. 4. The scheme shows the convergence of several signaling pathways ($SP_i$; i = 5) to a nodal point where the integration process of the various messages occurs. The output of the nodal point is a function of the integration process of the messages (f = $SP_i$). The figure shows also the potential importance of the temporal coding in the integration process by the different delays (and hence for the output) in the arrival of the various signals to the nodal point (see also Fig. 2).

lular signaling pathways, integrative units can be formed that very easily can be assembled and disassembled, according to the needs of the cell.

Thus, both WT and VT are used to create intracellular molecular circuits. For example, the MAPK cascade is mainly WT. However, the terminal MAPK, once activated, migrates (VT) into the nucleus where it phosphorylates and in this way activates transcription factors.

There are some potential arrangements of high functional significance to be considered. In fact, it can be hypothesized that the following instances are all theoretically possible:

- The WT signaling pathways are completely precabled and they need only to be activated at some nodal point (e.g., at the plasma membrane receptor site) to transmit (and/or elaborate) information.
- The WT signaling pathways are partially precabled and assembled when needed.
- The WT signaling pathways are to be entirely cabled.

The same logical scheme may hold for receptor mosaics. In fact, as discussed above, the following can be surmised.

- The receptor mosaic is already present at the plasma membrane level.
- The receptor mosaic is assembled from homo-heterodimers or simple oligomers, when needed.

These concepts may allow tackling, in a new way, some problems concerning the signaling processes within a cell and the generation of the cell response. In fact, by applying the concept of WT and VT and taking into proper consideration the hypothesis on the informational role of the receptor mosaic, it is possible to explain how the signaling pathways can operate with a limited number of components as follows:

- with selectivity of the signaling pathways, notwithstanding the fact that common signals and common protein modules are used in the various signaling pathways; and
- with a complexity of the biological response in spite of the activation of a single signaling pathway. In fact, as pointed out for neural networks, it is our opinion that it would be better to speak of "syndromic responses" rather than of a single response (elementary responses).
- In this way we can also understand the extraordinary capability of the cell to adapt to the highly variable challenging conditions in which the cell must live and operate in a finalistic fashion.

As shown in Fig. 1, the adaptation of the cell to challenges starts at the level of the membrane where the receptors can modify their conformation in response to the chemical-physical environments with which they are in contact. The binding of the neurotransmitter to its receptor (or of the neurotransmitters to the various types of receptors) will trigger the activation or modulation not only along one single signaling pathway but along several pathways. In this way the intracellular machinery is tuned in a form that can be described not with a "branch" (i.e., a single signaling pathway) but with a "tree" (see Fig. 2) where some main branches can be recognized but where the entire tree is important for the biological response of the cell (syndromic response). Selectivity is obtained since WT allows the direct and "private" link to the input site (e.g., the receptor) with the target (e.g., an enzyme).

It should be noted that in a nodal point (Fig. 4), it is possible to have summations as well subtractions of WT and VT signals. Thus, the output of the nodal point is the result of such an integration. Furthermore, it should be noted that the integration in the nodal point follows a temporal code that depends on the timing of the summations/subtractions among the different signals, since for each of these signals the progression toward the nodal point is different depending on the length and speed of its transmission along the respective signaling pathway.

A peculiar aspect of this process of integration at the nodal points is its dependence on the assembly/disassembly of the single modules (proteins) of the wire that forms the WT signaling pathway in question or on the characteristic of the diffusion of signals in the case of VT signaling pathways. In the case of WT, sometimes pieces of the wire can continue to operate: see, e.g., Raf-MEK-MAPK that goes on to operate also after Raf dissociates from Ras-GDP (Egan and Weinberg, 1993).

This possibility of assembling prewired segments of the various signaling pathways could, at least in part, explain the extraordinary capability of the cell to promptly adapt its responses to highly variable challenging conditions.

*D. Receptor/Receptor Interactions in the Striatopallidal GABA Neurons: Implications for Parkinson's Disease, Schizophrenia, and Drug Addiction*

*1. Localization of Adenosine $A_{2A}$, Dopamine $D_2$, and Glutamate Metabotropic $mGluR_5$ Receptors in the GABA Striatopallidal Neuron.* The striatum is the main input structure of the basal ganglia and a key component of the motor and limbic systems. On the basis of its afferent and efferent connections the striatum is currently subdivided into two parts, the dorsal and the ventral striatum. Accordingly, the dorsal striatum is mainly represented by the dorsolateral part of the nucleus caudate putamen and the ventral striatum is made of the ventromedial caudate putamen, the nucleus accumbens (with its two compartments, shell and core) and the olfactory tubercle (Heimer et al., 1995; Gerfen and Wilson, 1996). The basic intrastriatal circuitry is quite simple, only one type of neuron, the projecting GABAergic neuron, constitutes more that 90% of the striatal neuronal population. The GABAergic efferent neurons mainly convey information carried by dopaminergic mesencephalic cells, which are located in the substantia nigra and ventral tegmental area, and by glutamatergic cells located in cortical and limbic areas, like the amygdala and hippocampus (Heimer et al., 1995; Gerfen and Wilson, 1996) (Fig. 5).

There are two subtypes of striatal GABAergic efferent neurons that give rise to the two dorsal striatal efferent systems, which connect the dorsal striatum with the output structures of the basal ganglia, the substantia nigra pars reticulata, and the entopeduncular nucleus (Alexander and Crutcher, 1990; Heimer et al., 1995; Gerfen and Wilson, 1996). These are called direct and indirect pathways. The direct pathway is made of striatonigral and striatoentopeduncular neurons. The indirect pathway consists of the striatopallidal GABAergic neurons, pallido-subthalamic GABAergic neurons, and glutamatergic neurons, which connect the subthalamic nucleus with the output structures. Pallidal GABAergic neurons also project directly to the output structures without using the subthalamic nucleus relay (Fig. 5). The striatopallidal GABAergic neurons contain the pep-
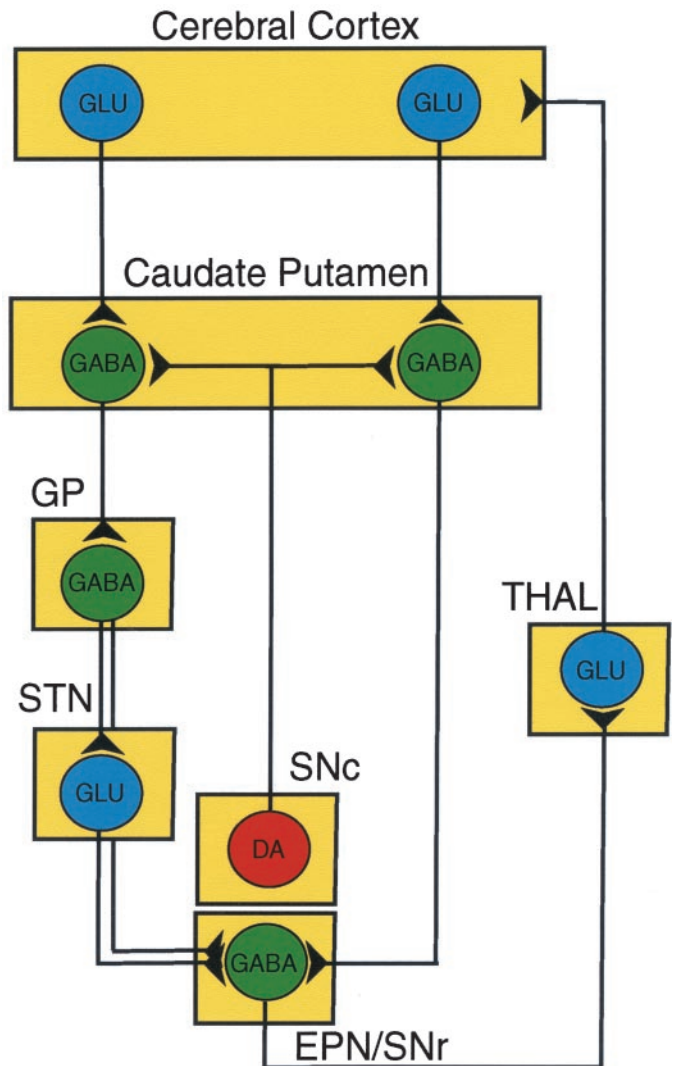


FIG. 5. Scheme of the basal ganglia circuitry involving the caudate-putamen as the striatal component and showing the two striatal efferent pathways (see text). DA, dopaminergic neuron; GABA, GABAergic neuron; GLU, glutamatergic neuron; EPN, entopeduncular nucleus (internal segment of the globus pallidus in primates); GP, globus pallidus (external segment of the globus pallidus in primates); SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; THAL, thalamus.

tide enkephalin and dopamine receptors predominantly of the $D_2$ subtype. On the other hand, the striatonigral and striatoentopeduncular GABAergic neurons contain the peptides dynorphin and substance P and dopamine receptors predominantly of the $D_1$ subtype. Stimulation of the direct pathway results in motor activation and stimulation of the indirect pathway produces motor inhibition. Dopamine, or dopamine agonists, will induce motor activation by activating the direct pathway (acting on stimulatory $D_1$ receptors) and by depressing the indirect pathway (acting on inhibitory $D_2$ receptors) (Alexander and Crutcher, 1990; Gerfen and Wilson, 1996). In Parkinson's disease a preferential degeneration of the nigrostriatal dopaminergic system produces striatal dopamine depletion with the consequent impairment of the functioning of these circuits, which is associated with

hypokinesia. Hyperactivity of the GABAergic striatopallidal neurons due to the release from the strong $D_2$ receptor-mediated tonic inhibitory effects of endogenous dopamine is probably the main pathophysiological mechanism responsible for this hypokinesia (Obeso et al., 2000). The consequent increased neuronal activity of the subthalamic nucleus and the output structures of the basal ganglia (in particular the internal segment of the globus pallidus, which is the counterpart of the rat entopeduncular nucleus in primates) seems to be the functional hallmark of the parkinsonian state, that gives the basis of the surgical treatment in this disease (lesion of the subthalamic nucleus or pallidotomy) (Obeso et al., 2000).

The same two subtypes of GABAergic neurons, with the same segregation of dopamine receptor subtypes are also found in the ventral striatum (Le Moine and Bloch, 1995). However, the organization of the targets of the ventral striatum is different from its dorsal counterpart (Heimer et al., 1995). Although the ventral striatum sends projections to the pallidal complex (ventral pallidum), the entopeduncular nucleus and the substantia nigra-ventral tegmental area, it also sends projections to the extended amygdala, the lateral hypothalamus and the lateral mesopontine tegmental nucleus. The ventral GABAergic striatopallidal neuron plays a key role in the conversion of motivation into action via transfer of information from the limbic to the motor system (for review, see Ferré, 1997). In fact, it is a final common pathway for opiate- and psychostimulant-mediated reward (Koob, 1999) and a common target for antipsychotic drugs (Ferré, 1997). Thus, blockade of $D_2$ receptors in the ventral GABAergic striatopallidal neuron seems to be involved with the antipsychotic effects of neuroleptics or, at least, with their therapeutic effect on the positive symptoms of schizophrenia. On the other hand, blockade of $D_2$ receptors in the dorsal GABAergic striatopallidal neuron is associated with the extrapyramidal side effects of neuroleptics, such as parkinsonism. One of the possible properties of atypical neuroleptics, which have a decreased liability of those side effects, is a preferential $D_2$ receptor blockade in the ventral striatum (reviewed in Ferré, 1997).

In addition to dopamine and glutamate, which are believed to have opposite actions on the striatal GABAergic efferent neurons (Carlsson and Carlsson, 1990), adenosine is a very important modulator (Snyder 1985) that plays an important integrative role in the function of these neurons (Ferré et al., 1997; Fuxe et al., 1998) (Fig. 6). Extracellular adenosine depends mostly on the intracellular concentration of adenosine and nucleotides, such as ATP, AMP, and cAMP (Latini and Pedata, 2001). In some brain areas, like the hippocampus, most of the extracellular adenosine seems to depend mostly on intracellular adenosine, the concentration of which depends on the rate of breakdown and synthesis of ATP (Latini and Pedata, 2001). Thus, in this case,
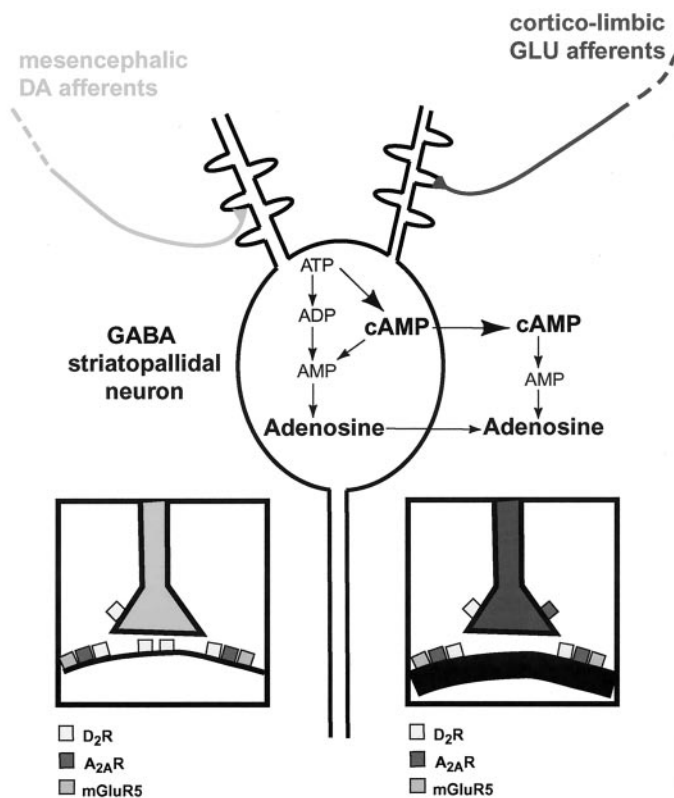


FIG. 6. Localization of dopamine $D_2$ receptors, adenosine $A_{2A}$ receptors, and metabotropic $mGluR_5$ receptors in the GABAergic striopallidal neuron. The three receptors are most probably colocalized in a perisynaptic ring surrounding both symmetric synapses (including dopaminergic synapses; left square) and asymmetric synapses (glutamatergic synapses; right square) (see text). The main sources of extracellular striatal dopamine [mesencephalic dopaminergic (DA) afferents] glutamate [cortico-limbic glutamatergic (GLU) afferents], and adenosine (intracellular adenosine and extracellular cAMP) are also indicated.

different from what happens with classical neurotransmitters, which are mostly released by the nerve terminals in response to the arrival of the impulse flow, adenosine is released as a neuromodulator (Snyder, 1985) by the effector cells in response to an increased metabolic demand (Ferré and Fuxe, 2000). However, in the striatum, it has recently been suggested that the main source of extracellular adenosine is extracellular cAMP (Manzoni et al., 1998), which is metabolized to AMP by means of phosphodiesterases and then to adenosine by the ectoenzyme 5′-nucleotidase. Since cAMP can only be generated intracellularly by the action of the enzyme adenylyl cyclase, striatal extracellular adenosine would mostly reflect an increased activation of receptors positively linked to adenylyl cyclase. The actions of adenosine are mediated by specific G protein-coupled receptors. From the four subtypes of adenosine receptors so far identified ($A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ receptors) $A_1$ receptors and, particularly, $A_{2A}$ receptors are specially concentrated in the striatum (Fredholm, 1995a,b; Svenningsson et al., 1999b; Fredholm et al., 2001). $A_{2A}$ receptors are mostly localized in both dorsal and ventral GABAergic striatopallidal neurons, colocalized with dopamine $D_2$ receptors (Schiffmann et al., 1991, 1993; Fink

et al., 1992; Svenningsson et al., 1997, 1999b). On the other hand, adenosine $A_1$ receptors are localized in all the striatal neuronal elements and dopamine $D_1$ receptors in the GABAergic striatonigral-striatoentopeduncular neurons are colocalized with adenosine receptors of the $A_1$ subtype (Ferré et al., 1996).

An important amount of evidence exists for the existence of specific antagonistic $A_{2A}/D_2$ receptor interactions that modulate the function of GABAergic striatopallidal neurons (Ferré et al., 1993a, 1997; Fuxe et al., 1998). Furthermore, specific antagonistic $A_1/D_1$ receptor interactions modulate the function of GABAergic striatonigral-striatoentopeduncular neurons (Ferré et al., 1997; Fuxe et al., 1998). It has been suggested that these receptor/receptor interactions are involved in the motor effects of adenosine agonists and antagonists (like the nonselective adenosine antagonist caffeine) and that they can provide a new therapeutic approach for the treatment of basal ganglia disorders and schizophrenia (Ferré et al., 1992, 1994a,b, 1997, 2001; Ferré, 1997; Lillrank et al., 1999; Fuxe et al., 2001). Here we will review the more relevant findings on the $A_{2A}/D_2$ receptor interactions as well as the recent data on the multiple interactions between these receptors and the group I metabotropic glutamate receptor $mGluR_5$.

Glutamate acts on both ionotropic and metabotropic G protein-coupled receptors. Molecular and pharmacological characterization studies have currently divided the metabotropic glutamate receptor family into three groups (I to III) (Pin and Duvoisin, 1995; Bockhaert and Pin, 1999; Hermans and Challiss, 2001). Group I includes $mGluR_1$ and $mGluR_5$ receptors, the latter of which is particularly expressed in the striatum, especially in the striatal GABAergic efferent neurons (Shigemoto et al., 1993; Testa et al., 1995; Tallaksen-Green et al., 1998). The ultrastructural analysis of the localization of $mGluR_5$ receptors in the striatopallidal complex in primates demonstrated that $mGluR_5$ receptor immunoreactivity is commonly found perisynaptically to asymmetric (glutamatergic) postsynaptic synapses (Fig. 6) but also at symmetric synapses formed by tyrosine hydroxylase-immunoreactive (dopaminergic) terminals (Smith et al., 2000). The recent ultrastructural analysis of the localization of $A_{2A}$ receptors in the rat striatum showed a very similar localization to that described for $mGluR_5$ receptors in primates. Thus, $A_{2A}$ receptors are mostly localized postsynaptically in the dendrites and dendritic spines of the striatal GABAergic neurons. Importantly, $A_{2A}$ receptor-immunoreactivity was observed primarily at asymmetric synapses (Rosin et al., 1998; Hettinger et al., 2001). Although less frequently $A_{2A}$ receptor immunoreactivity was also found presynaptically at asymmetric synapses and postsynaptically at symmetric synapses (Hettinger et al., 2001). Finally, in the rat striatum $D_2$ receptor immunoreactivity has been found both presynaptically (in both dopaminergic and glutamatergic terminals) and postsynaptically. At the postsynaptic level, $D_2$ receptor immunoreactivity has not only been observed at symmetric synapses but also perisynaptically at asymmetric synapses (Yung et al., 1995; Delle Donne et al., 1997). In summary, these morphological findings strongly suggest that the three receptors, $A_{2A}$, $mGluR_5$, and $D_2$, are colocalized in the striatopallidal GABAergic efferent neuron, postsynaptically and perisynaptically at dopaminergic and glutamatergic synapses (Fig. 6). Dopamine and glutamate may be able to reach this localization, not only by spillover from the respective nerve terminals, but also by longer distance VT. Finally, the colocalization gives a morphological frame for the existence of multiple $A_{2A}/D_2/mGluR_5$ receptor interactions.

*2. Interactions between Adenosine $A_{2A}$, Dopamine $D_2$, and Glutamate Metabotropic $mGluR_5$ Receptors in the GABA Striatopallidal Neuron: Biochemical-Cellular Level.* As previously discussed, evidence for the existence of physical interactions between $A_{2A}$ and $D_2$ receptors and between $A_{2A}$ and $mGluR_5$ receptors has been obtained with coimmunprecipitation experiments in cell lines that express, constitutively or after stable or transient cotransfection, the corresponding receptors (Ferré et al., 2002; Hillion et al., 2002). $A_{2A}/mGluR_5$ heteromeric complexes have also been demonstrated in rat striatal membrane preparations with coimmunoprecipitation experiments (Ferré et al., 2002). Different behavioral and biochemical models have demonstrated the existence of functionally significant antagonistic $A_{2A}/D_2$ receptor and $mGluR_5/D_2$ receptor interactions and synergistic $A_{2A}/mGluR_5$ receptor interactions. At the biochemical level, by using membrane preparations of rat striatum or of cell lines expressing the corresponding receptors and by using receptor autoradiography in rat and human striatal sections, it has been shown that stimulation of $A_{2A}$ receptors decreases the affinity of $D_2$ receptors for dopamine or dopamine agonists. These results were shown, e.g., as an increase in $K_H$ or $IC_{50}$ in competition experiments of dopamine versus a radioactively labeled $D_2$ receptor antagonist or as an increase in $K_D$ in saturation experiments with tritiated dopamine or quinpirole (Ferré et al., 1991d, 1994a,b, 1999a; Dasgupta et al., 1996a; Dixon et al., 1997; Lepiku et al., 1997; Kull et al., 1999; Franco et al., 2000; Salim et al., 2000; Díaz-Cabiale et al., 2001). In receptor autoradiography experiments, the $A_{2A}$ receptor-mediated modulation of $D_2$ receptor binding properties was found to be stronger in the ventral compared with the dorsal striatum (Ferré et al., 1994b; Díaz-Cabiale et al., 2001). Dopamine receptors of the $D_3$ subtype are structurally and pharmacologically very similar to $D_2R$ and they are specially concentrated in the ventral striatum (Missale et al., 1998). Therefore, it was suggested that the stronger $A_{2A}/D_2$ receptor interaction in the ventral striatum might be related to the action of $A_{2A}$ receptor agonists on $D_3$ receptors, in addition to $D_2$ receptors, as suggested by the demonstration of the $A_{2A}$ receptor agonist-mediated

modulation of $D_3$ receptor agonist binding in vivo (Hille-fors et al., 1999). Also, in membrane preparations from rat striatum, nonselective stimulation of group I gluta-mate metabotropic receptors or selective stimulation of $mGluR_5$ receptors decreased the affinity of $D_2$ receptors for dopamine (Ferré et al., 1999a; Rimondini et al., 1999, Popoli et al., 2001). Finally, costimulation of $A_{2A}$ recep-tors and group I glutamate metabotropic receptors or $mGluR_5$ receptors exerted a synergistic effect on the modulation of the binding characteristics of $D_2$ receptors (Ferré et al., 1999a; Rimondini et al., 1999; Popoli et al., 2001).

In addition to the intramembrane receptor/receptor interactions, the existence of $A_{2A}/D_2$ and $A_{2A}/mGluR_5$ heteromeric receptor complexes provides the possibility for close cross-talk between $A_{2A}$, $D_2$, and $mGluR_5$ recep-tors. This would synchronize the activation of these re-ceptors to interact at the second-messenger level and beyond. The major signal transduction pathway used by $A_{2A}$ receptors depends on the activation of adenylyl cy-clase, by means of $G_s/G_{olf}$ coupling (Kull et al., 1999, 2000). $A_{2A}$ receptor-mediated adenylyl cyclase activa-tion generates cAMP, which activates a cAMP-depen-dent protein kinase (PKA), which in turn regulates the state of phosphorylation of various substrate proteins (Fig. 7). One of those proteins, DARPP-32 (dopamine and cyclic adenosine 3′,5′-monophosphate-regulated phophoprotein, 32 kDa) is expressed in very high con-centration in the GABAergic efferent neurons (Green-gard et al., 1998, 1999). PKA-induced phosphorylation at a single site (Thr-34 of the rat sequence) converts DARPP-32 into a potent and selective inhibitor of pro-tein phosphatase-1 (PP-1) (Greengard et al., 1999). Fur-thermore, DARPP-32 is also phosphorylated to a high stoichiometry under basal conditions at another site, Thr-75, which converts it into an inhibitor of PKA (Nishi et al., 2000). PKA stimulates protein phosphatase-2A, which is the predominant phosphatase responsible for dephosphorylation of phospho-Thr-75-DARPP-32 (Nishi et al., 2000). It has been demonstrated that the removal of the inhibitory constraint on PKA by dephosphoryla-tion of phospho-Thr-75-DARPP-32 provides a mecha-nism of amplification of the PKA signal transduction pathway (Nishi et al., 2000). In striatal slices, Fisone and coworkers have demonstrated that stimulation of $A_{2A}$ receptors produces phosphorylation of DARPP-32 at Thr-34 and dephosphorylation of DARPP-32 at Thr-75 (Svenningsson et al., 1998, 2000; Lindskog et al., 1999, 2002). Another protein phosophorylated by PKA is the constitutive transcription factor CREB (cAMP response element-binding protein). Induction of cAMP liberates the catalytic subunits of PKA, which diffuse into the nucleus and induce cellular gene expression by phos-phorylating CREB at a serine residue (Ser-133) (Mayr and Montminy, 2001). CREB activity declines after a couple of hours of continuous stimulation, due to dephos-phorylation at Ser-133 by PP-1 (Mayr and Montminy,
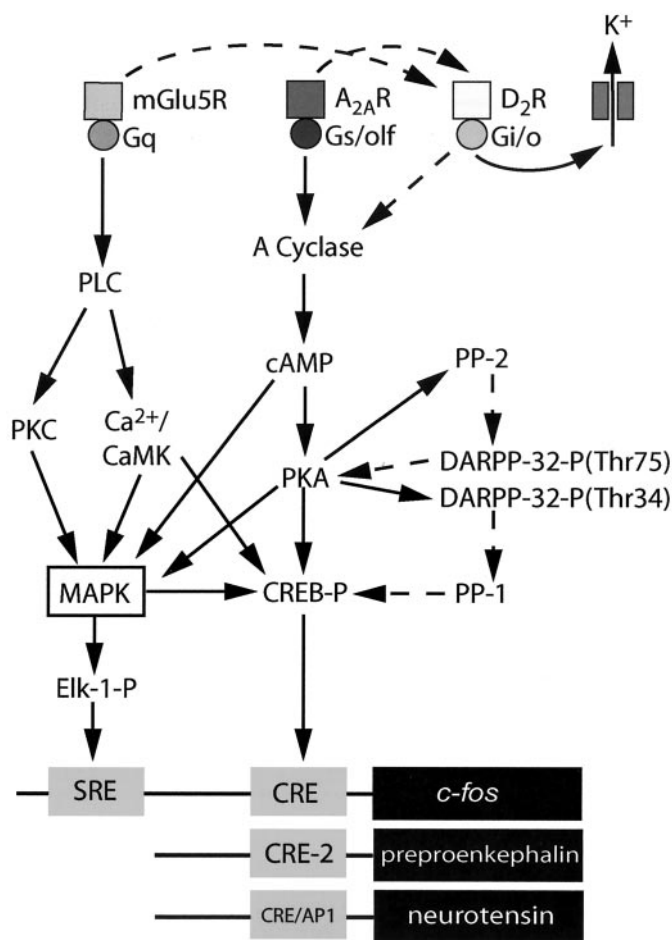


FIG. 7. Possible mechanisms responsible for the multiple biochemical interactions between dopamine $D_2$ receptors, adenosine $A_{2A}$ receptors, and metabotropic $mGluR_5$ receptors. Antagonistic $A_{2A}$ receptor/$D_2$ recep-tor and $mGluR_5/D_2$ receptor interactions have been demonstrated at the intramembrane level and an antagonistic $D_2/A_{2A}$ receptor interaction has been found at the adenylyl cyclase level. MAPKs seem to be the main coincidental signal involved in the synergistic $A_{2A}/mGluR_5$ interaction demonstrated at the level of c-*fos* expression. Furthermore, other levels of interaction upstream to MAPK could be the nonreceptor tyrosine kinase Src or other enzymes recently demonstrated to be activated by G protein-coupled receptors through G protein-independent signaling. This $A_{2A}/mGluR_5$ interaction allows the counteraction of the strong tonic $D_2$ re-ceptor-mediated inhibition of $A_{2A}$ receptor signaling. Broken arrows: antagonistic interactions or inhibitory effects (see text). A cyclase, adeny-lyl cyclase; DARPP-32 (dopamine and cAMP-regulated phosphoprotein, 32 kDa); DARPP-32-P(Thr-34) and DARPP-32-P(Thr-75): DARPP-32-phosphorylated at threonine residues 34 and 75, respectively.

2001). Thus, through PKA activation $A_{2A}R$ stimulation in the GABAergic striatopallidal neurons can potentially produce a sustained increase in the transcription of some CREB-modulated genes by a mechanism involving increased CREB phosphorylation and decreased CREB dephosphorylation (through phospho-Thr-34-DARPP-32-mediated inhibition of PP-1 activity) (Fig. 7). The immediate-early gene c-*fos* and the preproenkephalin and neurotensin genes are very well studied target genes, whose promoters contain consensus sites for CREB-P binding (Borsook and Hyman, 1995; Evers et al., 1995; Hughes and Dragunow, 1995; Le et al., 1997; Herdegen and Leah, 1998). Recent studies have shown

that $A_{2A}$ receptor stimulation can, under certain conditions, increase the expression of c-fos (and other immediate-early genes), preproenkephalin, and neurotensin genes (Fig. 7) (see below).

One of the main fully documented signaling effects of $D_2$ receptors is adenylyl cyclase inhibition by means of its coupling to $G_{i/o}$ proteins (for review, see Missale et al., 1998). Also dependent on $G_{i/o}$ coupling, but independent of cAMP, $D_2$ receptors have been shown to modulate the activity of $K^+$ channels. In many preparations, including acutely dissociated striatal neurons (Freedman and Weight, 1988), $D_2$ receptor stimulation has been shown to increase outward $K^+$ currents, leading to cell hyperpolarization (Missale et al., 1998). Furthermore, other less well characterized cAMP-independent signaling pathways used by $D_2$ receptors, such as activation of PLC, inhibition of inward $Ca^{2+}$ currents, arachidonic acid release, inhibition of $Na^+$-$K^+$-ATPase and MAPK activation, have been reported (Missale et al., 1998; Yan et al., 1999). A strong antagonistic interaction between $A_{2A}$ receptors and $D_2$ receptors at the adenylyl cyclase level has been demonstrated in different cell lines (Kull et al., 1999; Hillion et al., 2002) (Fig. 7). In CHO cells stably cotransfected with $A_{2A}$ and $D_2$ receptor cDNAs, stimulation of $A_{2A}$ receptors produced a strong stimulation of cAMP, CREB phosphorylation and increased c-fos expression (Kull et al., 1999). A selective $D_2R$ agonist dose dependently counteracted these effects and a complete blockade was attained at low concentrations of the $D_2R$ agonist (Kull et al., 1999). Similarly, in striatal slices a $D_2$ receptor agonist completely counteracted Thr-34 phosphorylation of DARPP-32 by $A_{2A}$ receptor stimulation (Lindskog et al., 1999).

In the striatum, $D_2$ receptors are tonically stimulated by basal endogenous levels of dopamine (see above). Many experimental results indicate that this tonic $D_2$ receptor stimulation strongly counteracts a tonic $A_{2A}$ receptor stimulation induced by the basal striatal levels of adenosine. In this way, the products of $A_{2A}$ receptor activation are kept at low concentration under normal basal conditions and inactivation of striatal $D_2$ receptor-mediated neurotransmission (by administration of $D_2$ receptor antagonists, striatal dopamine depletion or genetic $D_2$ receptor inactivation) liberates $A_{2A}$ receptor-mediated function from the strong $D_2$ receptor-mediated tonic inhibition. This results in a very significant increase in the striatal expression of c-Fos, Thr-34-phosphorylated DARPP-32, enkephalin, and neurotensin, which is partially counteracted by genetic inactivation or pharmacological blockade of $A_{2A}$ receptors (Schiffman and Vanderhaeghen, 1993; Morelli et al., 1995; Pollack and Fink, 1995; Boegman and Vincent, 1996; Adams et al., 1997; Pinna et al., 1997, 1999; Richardson et al., 1997; Svenningsson et al., 1999a, 2000; Ward and Dorsa, 1999; Chen et al., 2000, 2001; Zahniser et al., 2000; Dassesse et al., 2001; Ferré et al., 2002; see Section I.B.). Since also at the behavioral level $A_{2A}$ receptor

antagonists counteract the motor depressant and cataleptic effects secondary to the genetic inactivation or pharmacological interruption of $D_2$ receptor-mediated neurotransmission (see below), these results strongly suggest that an important part of the biochemical and behavioral effects induced by interruption of $D_2$ receptor-mediated neurotransmission are due to the liberation of $A_{2A}$ receptor signaling. This can have obvious implications for the possible application of $A_{2A}$ receptor antagonists in Parkinson's disease. On the other hand, these results also suggest that the biochemical effects related to adenylyl cyclase activation (which are only clearly apparent when the $D_2$ receptor-mediated inhibitory tone is removed) do not play a major role in some functional and behavioral effects produced by the administration of $A_{2A}$ receptor agonists and antagonists (see below). For instance, the systemic administration of a low dose of a selective $A_{2A}$ receptor agonist produces a pronounced motor depression (already shown to be centrally mediated; see Barraco et al., 1993) and selectively counteracts $D_2$ receptor agonist-mediated behaviors (Rimondini et al., 1997, 1998) without inducing an increased c-fos striatal expression (Morelli et al., 1995; Pinna et al., 1997; Ferré et al., 2002; see Section I.B.2.k.). Most probably those behavioral effects are related to the reciprocal antagonistic $A_{2A}/D_2$ receptor intramembrane interaction, which can be more effective at modulating other $D_2$ receptor-mediated signaling pathways, such as the opening of $K^+$ channels.

As previously mentioned, some studies suggest that a preferential blockade of $D_2$ receptors in the ventral, compared with the dorsal striatum, is one of the main factors responsible for the atypical profile of an antipsychotic. Some of these studies are based on the differential increase in c-fos expression in the different striatal compartments. Thus, typical antipsychotics, like haloperidol, increase c-fos expression in the dorsal and ventral striatum, whereas atypical antipsychotics, like clozapine, selectively elevates c-fos expression in the ventral striatum, and especially in the shell of the nucleus accumbens (Deutch et al., 1992; Robertson and Fibiger, 1992; Merchant and Dorsa, 1993; Pinna et al., 1999). A similar differential response to haloperidol and clozapine has also been demonstrated for the striatal expression of neurotensin (Merchant and Dorsa, 1993). It has also been shown that the increased c-fos expression in the ventral striatum induced by both haloperidol and clozapine selectively takes place in the ventral GABAergic striatopallidal neuron (Robertson and Jian, 1995) and that it can be counteracted by the systemic administration of an $A_{2A}$ receptor antagonist (Pinna et al., 1999). Therefore, these results suggest that $A_{2A}$ receptors might be involved in the mediation of the antipsychotic effects of neuroleptics (see below).

It remains to be determined which are the physiological conditions (without interruption of $D_2$ receptor-mediated neurotransmission) that would allow $A_{2A}$ recep-

tor stimulation to produce a clear adenylyl cyclase activation, with the corresponding increase in Thr-34 DARPP-32 and CREB phosphorylation and increase in the expression of c-*fos* and the preproenkephalin and neurotensin genes. One such condition seems to be mGluR$_5$ receptor coactivation (Ferré et al.,. 2002). The major signal transduction pathway used by mGluR$_5$ receptors (through G$_q$ proteins) is activation of PLC, which releases IP$_3$ and diacylglycerol, which cause the release of intracellular Ca$^{2+}$ and the activation of PKC, respectively (Pin and Duvoisin, 1995; Hermans and Challis, 2001) (Fig. 7). In HEK-293 cells transiently cotransfected with A$_{2A}$ and mGluR$_5$ receptors, stimulation of A$_{2A}$ receptors produced an increase in cAMP levels that was not significantly modified by the concomitant stimulation of mGluR$_5$ receptors (Ferré et al., 2002). As expected, stimulation of mGluR$_5$ receptors induced an increase in [Ca$^{2+}$]$_i$. In addition to mGluR$_5$ receptor stimulation, a selective A$_{2A}$ receptor agonist also produced a significant increase in [Ca$^{2+}$]$_i$ in cotransfected cells, which would agree with recent studies in rat striatal slices which suggest that A$_{2A}$ receptors can also use PLC/IP$_3$ signaling under certain conditions (Wirkner et al., 2000). However, at the level of [Ca$^{2+}$]$_i$ an absence of synergistic A$_{2A}$/mGluR$_5$ receptor interaction was observed (Ferré et al.,. 2002). The lack of synergistic interaction at the second messenger level (cAMP and Ca$^{2+}$) was somehow surprising in view of previous studies showing synergistic effects on cAMP accumulation of group I glutamate metabotropic receptors and receptors positively linked to adenylyl cyclase in neuronal primary cultures (Cartmell et al., 1998; Paolillo et al., 1998) and in view of recent findings on synergistic effects between A$_1$ receptors and mGluR$_{1\alpha}$ receptors on [Ca$^{2+}$]$_i$ in cotransfected HEK-293 cells (Ciruela et al., 2001). Nevertheless, a strong functional synergistic A$_{2A}$/mGluR$_5$ receptor interaction on MAPK (ERK 1/2) and on c-*fos* expression was found in cotransfected cells (Ferré et al.,. 2002). Accordingly, a significant striatal c-*fos* induction was obtained after the concomitant central administration of A$_{2A}$ and mGluR$_5$ receptor agonists, which were ineffective when administered alone (Ferré et al., 2002). This strongly suggests that concomitant stimulation of A$_{2A}$ and mGluR$_5$ receptors is one of the mechanisms by which A$_{2A}$ receptor stimulation can overcome the tonic inhibitory effect of dopamine and induce striatal c-*fos* expression. We favor the idea that this mechanism can take place under conditions of intense glutamatergic neurotransmission, which is known to induce adenosine release, most probably, due to the neuronal metabolic demand imposed by the increased excitatory input (Ferré and Fuxe, 2000; Latini and Pedata, 2001).

The protein encoded by c-*fos* (c-Fos) is an inducible transcription factor, expression of which is controlled by pre-existing constitutive transcription factors, such as CREB, serum response factor, and TCF/Elk-1 proteins (Herdegen and Leah, 1998; Ches and Wang, 2001). Upon

phosphorylation, CREB and TCF/Elk-1 (which requires serum response factor) activate c-*fos* transcription by binding to the CRE and serum response element regulatory elements of the c-*fos* promoter, respectively (Hughes and Dragunow, 1995; Herdegen and Leah, 1998) (Fig. 7). It is generally accepted that CREB is mostly phosphorylated by PKA (see above) whereas TCF/Elk-1 is mostly phosphorylated by MAPK (Vanhoutte et al., 1999). In the case of group I metabotropic receptors, MAPK activation seems to involve PKC (Ferraguti et al., 1999) and Ca$^{2+}$/calmodulin-dependent protein kinases (CaMK) (Choe and Wang, 2001). However, recent studies indicate that both MAPK and CREB can be convergent targets for different elements of the cAMP/PKA and the PLC/PKC-CaMK signaling pathways (Fig. 7) (Impey et al., 1999; Sweatt, 2001; Wu et al., 2001). Since the A$_{2A}$/mGluR$_5$ receptor synergistic effect on c-*fos* expression found in cotransfected cells was completely counteracted by a ERK 1/2 kinase inhibitor (Ferré et al., 2002), MAPK seems to be a main biochemical integration element responsible for the synergistic interactions between A$_{2A}$ and mGluR$_5$ receptors. Furthermore, other levels of interaction upstream to MAPK could be the nonreceptor tyrosine kinase Src or other enzymes recently demonstrated to be activated by G protein-coupled receptors through G protein-independent signaling (Heuss and Gerber, 2000). Given the key role of immediate-early genes in the coupling of early neuronal responses to long-term adaptive changes (Hughes and Dragunow, 1995; Berke and Hyman, 2000), those results suggest that A$_{2A}$/mGluR$_5$ receptor interactions might be involved in striatal neuronal plasticity. More specifically, this mechanism may underlie the recently described dopamine-independent increased c-*fos* expression in the striatopallidal neurons associated with sensitization to psychostimulants (Uslaner et al., 2001). In fact, both striatal A$_{2A}$ and mGluR$_5$ receptors have recently been shown to be involved in certain forms of striatal plasticity, including cortico-striatal long-term potentiation and long-term depression (d'Alcantara et al., 2001; Sung et al., 2001).

*3. Interactions between Adenosine A$_{2A}$, Dopamine D$_2$, and Glutamate Metabotropic mGluR$_5$ Receptors in the GABA Striatopallidal Neuron: Physiological-Behavioral Level.* The first functional approach used to study the interactions between adenosine and dopamine receptors in the GABAergic striatopallidal neurons was the dual-probe in vivo microdialysis approach in freely moving rats. In these experiments, one microdialysis probe is implanted in the caudate-putamen or the nucleus accumbens, and the second one is implanted in the ipsilateral globus pallidus or the ipsilateral ventral pallidum, respectively. This allows determining in freely moving animals the modulation of GABAergic neurotransmission in the dorsal and ventral striatopallidal neurons (Ferré et al., 1993a, 1994a; Díaz-Cabiale et al., 2002b). With these experiments, a main difference in the effects

of $D_2$ receptor stimulation was found between the dorsal and the ventral striatopallidal pathways. The striatal perfusion of a $D_2$ receptor agonist decreased the extracellular levels of GABA in the globus pallidus, but not in the ventral pallidum. On the other hand, $D_2$ receptor antagonists increased GABA levels in the ventral pallidum and did not significantly modify GABA levels in the globus pallidus (Ferré et al., 1993a, 1994a; Díaz-Cabiale et al., 2002b). Also, a different effect was obtained with the striatal perfusion of an $A_{2A}$ receptor agonist. In this case, GABA levels were only increased in the ventral pallidum (Ferré et al., 1993a, 1994a; Díaz-Cabiale et al., 2002b). Nevertheless, recent studies by Ochi et al. (2000) have also found a significant increase in the GABA levels of the globus pallidus after the striatal infusion (through an injection needle) of an $A_{2A}$ receptor agonist. The reciprocal $A_{2A}/D_2$ receptor interactions could also be demonstrated in microdialysis experiments. Thus, the $D_2$ receptor agonist-induced decrease and the $A_{2A}$ receptor agonist-induced increase in pallidal levels could be counteracted by the striatal coperfusion with $A_{2A}$ and $D_2$ receptor agonists, respectively (Ferré et al., 1994a; Díaz-Cabiale et al., 2002b). Altogether, these results suggest the existence of functional regional differences in the $A_{2A}/D_2$ receptor interactions, with a relatively stronger $A_{2A}$ receptor signaling in the ventral compared with the dorsal striatum, in agreement with some biochemical (Ferré et al., 1994a; Pinna et al., 1997; Díaz-Cabiale et al., 2002b), and behavioral studies (see below). We have hypothesized that these differences in the $A_{2A}/D_2$ receptor interactions between both striatal compartments might explain the atypical antipsychotic profile of adenosine $A_{2A}$ receptor agonists in animal models (see below). As mentioned earlier, this differential effect may be related to the existence of an additional antagonistic $A_{2A}/D_3$ receptor interaction in the ventral striatum (Diaz-Cabiale et al., 2001). Finally, in recent microdialysis experiments we have found that the perfusion of a selective $mGluR_5$ receptor agonist in the nucleus accumbens produces an increase in the extracellular levels of GABA in the ventral pallidum. Furthermore, coperfusion with an $A_{2A}$ receptor agonist produced a strong synergistic increase in pallidal GABA levels (Díaz-Cabiale et al., 2002b). From these results, we have suggested a possible use of $mGluR_5$ receptor agonists as antipsychotic drugs, alone or in combination with $A_{2A}$ receptor agonists.

In vitro studies with micropunctures of rat globus pallidus have shown that an antagonistic $A_{2A}/D_2$ receptor interaction also modulates GABAergic neurotransmission in the terminals of GABAergic striatopallidal neurons, which also express both $A_{2A}$ and $D_2$ receptors (Mayfield et al., 1996). In this experimental preparation, stimulation of pallidal $A_{2A}$ and $D_2$ receptors stimulates and inhibits GABA release, respectively, and the $D_2$ receptor-mediated inhibition of GABA release is counteracted by $A_{2A}$ receptor stimulation (Mayfield et al.,

1996). The $A_{2A}$ receptor-mediated stimulation of pallidal GABA release has been recently confirmed with in vivo microdialysis experiments (Ochi et al., 2000). However, experiments using striatal and pallidal synaptosomal preparations have given contradictory results. Thus, in these preparations, $A_{2A}$ receptor stimulation inhibited high KCl-stimulated GABA release (Kurokawa et al., 1994). Similarly, results obtained from experiments using intracellular and whole-cell patch-clamp recording in striatal slices suggested that striatal presynaptic $A_{2A}$ receptors exert an inhibitory modulation of GABA release (Mori et al., 1996). Based on these findings, Richardson et al. (1997) proposed that the main mechanisms by which $A_{2A}$ receptors would influence the function of striatal GABAergic neurons would be by a presynaptic inhibitory modulation of GABA release from their collateral recurrent axons. At this point, this theory is incompatible with the bulk of new information about the ultrastructural localization and function of striatal $A_{2A}$ receptors (see above).

Many behavioral studies have shown that in animals with intact striatal dopamine innervation, $A_{2A}$ receptor agonists behave as $D_2$ receptor antagonists (Heffner et al., 1989; Ferré et al., 1991a,b; Popoli et al., 1994; Kafka and Corbett, 1996; Hauber and Munkle, 1997; Rimondini et al., 1997, 1998; Wardas et al., 1999; Poleszak and Malec, 2000). Furthermore, although questioned by some studies (Kafka and Corbett, 1996; Hauber and Munkle, 1997), there are some behavioral data supporting a preferential effect of $A_{2A}$ receptor agonists in the ventral striatum (Barraco et al., 1993; Rimondini et al., 1997, 1998). When considering commonly used tests to screen the effects of antipsychotics, it is generally accepted that blockade of dopamine receptors in the ventral striatum is mostly responsible for the counteracting effects of the motor activation induced by novel stimuli (exploratory activity in a novel environment) and psychostimulants [such as amphetamine and phencyclidine (PCP)]. On the other hand, the counteraction of dopamine agonist-induced stereotypes and the induction of cataleptic immobility are believed to be mainly mediated by blockade of dopamine receptors in the dorsal striatum (reviewed in Ögren, 1996). The systemic administration of an $A_{2A}$ receptor agonist gave an atypical antipsychotic profile in these animal models, since it counteracted the motor-activating effects of psychostimulants at lower doses than those needed to counteract dopamine agonist-induced stereotypes or to induce catalepsy (Rimondini et al., 1997, 1998). In particular, very low doses of the $A_{2A}$ receptor agonist were necessary to counteract PCP-induced motor activity (Rimondini et al., 1997). In agreement, recent studies by Sills et al. (2001) have shown than an $A_{2A}$ receptor agonist can selectively reverse the reduction in prepulse inhibition of the acoustic startle response induced by PCP.

PCP is self-administered by humans and experimental animals (Carlezon and Wise, 1996; Jentsch and Roth,

1999), and its administration in humans reproduces both positive and negative symptoms of schizophrenia and exacerbates those symptoms in schizophrenic patients (Jentsch and Roth, 1999). Together with the neurochemical modifications induced by this drug, PCP administration is considered as a potential animal model of schizophrenia, and reversal of the effects of PCP is also commonly used as a model to screen for antipsychotic activity (Jentsch and Roth, 1999). The ventral striatopallidal GABA neurons are most probably the main neuronal target underlying the $A_{2A}$ receptor agonist-mediated counteraction of PCP-induced motor activation, since inhibition of ventral striatopallidal GABA transmission appears to be central in the mediation of some (rewarding) effects of PCP (Carlezon and Wise, 1996). Since the counteracting effect on PCP-induced motor activation is obtained with low doses of the $A_{2A}$ receptor agonist, which are not able to induce an increase in the striatal c-*fos* expression (see above), the antagonistic $A_{2A}/D_2$ receptor intramembrane interaction is most probably involved. In fact, the motor hyperactivity induced by PCP has been shown to be dependent on postsynaptic $D_2$ receptors and a high degree of $D_2$ receptor blockade is required to significantly counteract the stimulatory action of high doses of PCP (Ögren and Goldstein, 1994). Furthermore, the effect of $A_{2A}$ receptor stimulation was surmountable, and high doses of PCP could not be counteracted by the systemic administration of an $A_{2A}$ receptor agonist (Ferré et al., 2002; see *Section I.B.*). However, in agreement with the results obtained with striatal c-*fos* expression, when the $A_{2A}$ receptor agonist was coadministered with a mGluR$_5$ receptor agonist, which by itself did not produce any significant effect, PCP-mediated motor activation was significantly counteracted (Ferré et al., 2002). These results also fit very nicely with those obtained in microdialysis experiments (Díaz-Cabiale et al., 2002) and suggest that costimulation of $A_{2A}$ and mGluR$_5$ receptors can even counteract the effects induced by a strong stimulation of dopaminergic neurotransmission.

As previously mentioned, the systemic administration of $A_{2A}$ receptor antagonists counteracts most of the biochemical (see above) as well as the motor depressant and cataleptic effects secondary to the genetic inactivation or pharmacological interruption of $D_2$ receptor-mediated neurotransmission. This has been repeatedly demonstrated in a number of experimental models involving rodents pretreated with $D_2$ receptor antagonists, reserpine, or MPTP or after genetic inactivation of $D_2$ receptors (Casas et al., 1988; Popoli et al., 1991; Kanda et al., 1994; Giménez-Llort et al., 1995; Pollack and Fink, 1995; Malec, 1997; Shiozaki et al., 1999; Ward and Dorsa, 1999; Wardas et al., 1999; Aoyama et al., 2000; Chen et al., 2001; Ferré et al., 2001; Hauber et al., 2001) and involving MPTP-treated monkeys (Kanda et al., 1998a,b; Grondin et al., 1999). The results of these experiments suggest that $A_{2A}$ receptor antagonists can be

useful in the treatment of Parkinson's disease. However, although it has been claimed that $A_{2A}$ receptor antagonists might be devoid of the secondary dyskinetic effects associated with treatment with dopamine agonists (Kanda et al., 1998a,b; Grondin et al., 1999), it is still a matter of debate whether they can be useful as monotherapy or whether they would be more efficacious when combined with $D_2$ receptor agonists. In fact, in another classical experimental model of Parkinson's disease, the rat with a unilateral lesion of the nigrostriatal pathway, $A_{2A}$ receptor antagonists do not produce a turning behavior contralateral to the lesioned side (Fenu and Morelli, 1998; Strömberg et al., 2000; Ferré et al., 2001), which is a behavior that predicts antiparkinsonian activity (Ungerstedt, 1971). Nevertheless, in all the animal models of Parkinson's disease tested to date (reserpinized mice, rats with unilateral 6-OH-dopamine lesions, MPTP-treated monkeys), $A_{2A}$ receptor antagonists strongly potentiate the motor activation induced by $D_2$ receptor agonists (Ferré et al., 1991b, 2001; Jiang et al., 1993; Fenu et al., 1997; Grondin et al., 1999; Kanda et al., 2000; Koga et al., 2000; Popoli et al., 2000, 2001; Strömberg et al., 2000). Importantly, cotreatment with $A_{2A}R$ antagonists and L-dopa did not increase the non-wanted dyskinetic effects in MPTP-treated monkeys (Grondin et al., 1999; Kanda et al., 2000). Finally, recent electrophysiological experiments in the rat dopamine denervated striatum showed that the infusion of an $A_{2A}$ receptor antagonist did not produce any effect on its own, but strongly potentiated the $D_2R$ agonist-induced inhibition of striatal neuronal activity (Strömberg et al., 2000). Altogether, these results suggest that some pharmacological and, maybe, therapeutical effects of $A_{2A}$ receptor blockade can only be observed with the concomitant stimulation of $D_2$ receptors. This is also in agreement with some results obtained with $D_2$ receptor knockout mice where the motor effects of $A_{2A}$ receptor antagonists were attenuated (Chen et al., 2001). Thus, these experiments performed under complete inactivation of $D_2$ receptors demonstrate that some $A_{2A}$ receptor functions are dependent on the integrity of $D_2$ receptors and, most probably, on the integrity of $A_{2A}/D_2$ heteromeric receptor complexes. This is shown even more dramatically in recent studies by Zahniser et al. (2000), where a very significant functional uncoupling of $A_{2A}$ receptors (lack of $A_{2A}$ receptor agonist-induced GABA release in striatal/pallidal slices) was found in $D_2$ receptor knockout mice. On the other hand, a functional striatal hypodopaminergic activity (decreased striatal dopamine release and decreased psychostimulant-induced motor activation) has been found in mice lacking $A_{2A}$ receptors (Chen et al., 2000; Dassese et al., 2001). In this case, the possible role of a functional uncoupling of $D_2$ receptors remains to be determined.

Finally, the role of mGluR$_5$ and its interactions with $A_{2A}$ and $D_2$ receptors in animal models of Parkinson's disease is beginning to be evaluated. In unilaterally 6-OH-dopa-

mine-lesioned rats, the intracerebral administration of a selective mGluR$_5$ agonist selectively inhibited the contralateral turning induced by a D$_2$ receptor agonist (Popoli et al., 2001). The effect of the mGluR$_5$ agonist was potentiated by an A$_{2A}$ receptor agonist and attenuated by an A$_{2A}$ receptor antagonist (Popoli et al., 2001). These results suggest that a mGluR$_5$ antagonist, alone or in combination with A$_{2A}$ receptor antagonists and/or D$_2$ receptor agonists, might provide a new therapeutic approach for basal ganglia disorders, such as Parkinson's disease. In fact, recent studies by Ossowka et al. (2001) have found antiparkinsonian-like effects of mGluR5 antagonists in rats (Chase and Oh, 2000).

*4. Interactions between Adenosine, Dopamine, and Glutamate Metabotropic Receptors in the GABAergic Striatoentopeduncular and Striatonigral Neurons.* In these GABAergic efferent neurons of the striatum, which constitute the direct striatal efferent pathway (see above), evidence indicates that there might also exist multiple interactions between adenosine, dopamine, and group I metabotropic glutamate receptors. In this case, however, an antagonistic interaction between A$_1$ and D$_1$ receptors is involved (see above; Ferré et al., 1994b, 1996a,b, 1997, 1999b; Fuxe et al., 1998), which form heteromeric complexes (see above; Ginés et al., 2000). Although it remains to be demonstrated, most probably mGluR$_1$, instead of mGluR$_5$, receptors functionally interact with A$_1$ and D$_1$ receptors in these neurons, because of the demonstrated mGluR$_{1\alpha}$/A$_1$ heteromeric receptor complexes (see above; Ciruela et al., 2001). Furthermore, the demonstrated synergistic interactions in mGluR$_{1\alpha}$/A$_1$ heteromeric receptor complexes in cell lines with regard to agonist-induced increases in Ca$^{2+}$ signaling (Ciruela et al., 2001) are of interest. In view of the role of D$_1$ receptors in favoring motor initiation by excitatory effects on the direct pathway, the A$_1$/D$_1$/mGluR$_1$ receptor interaction might also have implications for the treatment of Parkinson's disease. Furthermore, A$_1$/D$_1$ receptor interactions can also have implications for schizophrenia and drug addiction, since similar to the A$_{2A}$/D$_2$ receptor interactions (see above), A$_1$/D$_1$ receptor interactions are stronger in the ventral compared with the dorsal striatum (Ferré et al., 1996b, 1999b; Mayfield et al., 1999).

## III. Implications of Receptor/Receptor Interactions for Drug Development

### A. The Ground for Novel Therapeutical Interventions

As pointed out above, receptor/receptor interactions are one type of protein/protein interactions, and they can occur in the context of various protein/protein interactions, i.e., inside of an aggregate of several proteins forming molecular circuits. This view is in agreement with the experimental evidence that, in almost all cases, proteins do not work alone but rather as part of larger complexes. The study of protein expression and interac-

tions is undertaken by "proteomics". Proteomics has been defined as the large-scale study of proteins encoded by a genome (Banks et al., 2000; Grant and Blackstock, 2001; Husi and Grant, 2001). Grant and colleagues have subdivided proteomics into "expression proteomics" and "functional (or interaction) proteomics" (Grant and Blackstock, 2001). Functional proteomics should analyze how proteins interact to form cellular machines. Thus, receptor/receptor interactions as well as the concept of cellular wiring (Pawson et al., 2001) made with protein modules (Pawson and Scott, 1997) can be part of the vast field of "functional proteomics".

This new way of looking at receptor activation and intracellular signaling pathways opens up the possibility of discovering molecular, physiological and pathological mechanisms until now unknown and also opens up an entire unexplored field for the development of drugs that should be aimed to specifically target protein/protein complex formation or to modulate the function of these protein complexes (Bond and Bouvier, 1998; Cochran, 2000). This field is just at its beginning (Tallman, 2000), and hence only the description of some of the most promising results until now obtained will be given.

It is convenient to subdivide the presentation as follows:

1. Analysis of drug action on protein/protein interactions
2. Possible targets for drugs acting on the heteromeric receptor complexes

### B. Theoretical Strategies to Target Receptor Complexes

From a theoretical standpoint at least three strategies can be employed to pharmacologically affect protein/protein interactions:

- To prevent the interaction by altering at least one of the two interacting protein interfaces (e.g., by a drug that localizes at the interfaces preventing the matching of the interface)
- To address the protein/protein interaction toward the formation of a different (inactive) complex (e.g., by favoring the matching between two different interfaces of the two partners)
- To favor the interaction of one of the two proteins toward the formation of a complex with another protein present in that compartment.

It should be underlined that to act in a predictable way on protein/protein interactions is a difficult task. Protein/protein interactions very often include discontinuous parts of the protein sequence. Thus, it is difficult to develop low molecular weight molecules capable of disrupting the protein interfaces that match together (Cochran, 2000). This difficulty seems to preclude the possibility to develop small molecules as pharmaceutical agents, but it should be kept in mind that low molecular weight is one of the features that determines better bioavailability.

The most straightforward strategy to develop a molecule that antagonizes a protein/protein interaction is that of reproducing the essential features of one of the two partner proteins in a smaller protein that, therefore, interferes with the complex formation. Some good results have been obtained in the case of interleukin-4 (Domingues et al., 1999). However, proteins even of a reduced size are still too large to be currently used as drugs. Thus, it is important to address the efforts toward using small peptides or, even better, peptidomimetic molecules as drugs (Cochran, 2000).

In a few cases it has been claimed that short peptides (10–20 amino acids), taken from a protein sequence, can disrupt the protein/protein interaction in which the parent protein is involved. However, according to Cochran these reports should be viewed with caution and carefully confirmed (Cochran, 2000).

In the field of peptides with a potential drug action an interesting case is that of an erythropoietin (EPO) agonist 20-residue peptide that has no resemblance with the natural hormone (so its affinity could not be predicted), but yet it binds to the hormone site as a dimer activating the receptor (Wrighton et al., 1996).

Another important approach in developing peptides that interfere with protein/protein interactions is that of replacing some natural amino acids with non-natural amino acids. This approach has been used to develop short peptides for the SH3 domains. These domains are very important for protein/protein interactions since they are small docking units present in many signal-transduction proteins (Pawson and Scott, 1997; Pawson et al., 2001). Thus, it has been possible to replace parts of the polyproline helix-recognition sequences with non-natural, *N*-substituted glycine residues (Nguyen et al., 1998). These peptide-peptoid hybrids often have higher affinity than that of the natural peptide and improved specificity for SH3 domains (Cochran, 2001) and by competing for binding to the SH3 domain they may stop the signal along the wiring pathways in the cytoplasm such as the RTK-Ras-MAP kinase pathway.

### C. Possible Targets for Drugs Acting on Heteromeric Receptor Complexes

When the receptor/receptor interactions are considered it is possible to make the following considerations:

- The receptor spans three different phases and in principle three different types of drugs could be developed according to where (extra-cellularly, intramembrane, intracellularly) the sequence of the receptor which should be targeted for the drug is located
- The receptor component of the three-component system that forms the GPCR appears as the target of choice for drugs, namely for two reasons: stoichiometry and accessibility of the target. The greater accessibility of a drug to the receptor component is evident. As far as the stoichiometry of the three

components is concerned it has been shown that the ratio of receptor/G-protein/adenylyl cyclase is in most instances equal to 1:100:3 (Ostrom et al., 2000). These data lead one to predict that the receptor or adenylyl cyclase are the best targets.

On the basis of these data and of the receptor/receptor interactions, it is possible to conceive of other pharmacological interventions besides the classical approach aimed to the activation or inhibition of the receptor due to occupation by the drug of the binding pocket for the natural ligand. Several other approaches are possible based on receptor/receptor interactions in heteromeric complexes:

- The drug is developed for one coreceptor to modulate another coreceptor at the recognition level (binding pocket). One example is to have an adenosine $A_{2A}$ receptor antagonist acting on the $A_{2A}$ coreceptor in the antagonistic $A_{2A}/D_2$ heteromeric receptor complex to produce enhancement of $D_2$ coreceptor signaling by removal of the $A_{2A}$-induced reduction of affinity of the $D_2$ coreceptor. Such a drug may become a novel antiparkinsonian drug with less side-effects as indicated from early (Fuxe and Ungerstedt, 1974; Fredholm et al., 1983; Herrera-Marschitz et al., 1988; Casas et al., 1988; Popoli et al., 1991 Jiang et al., 1993; Kanda et al., 1994) and recent work (Malec, 1997; Kanda et al., 1998a,b; Grondin et al., 1999; Shiozaki et al., 1999; Ward and Dorsa, 1999; Wardas et al., 1999; Aoyama et al., 2000; Strömberg et al., 2000; Chen et al., 2001; Ferré et al., 2001; Fuxe et al., 2001; Hauber et al., 2001; Pinna et al., 2001; Morelli and Wardas, 2001; T. Chase, personal communication). The best approach will be to block selectively the $A_{2A}$ binding pocket in the heteromeric complex and not those $A_{2A}$ receptors not linked to the $D_2$ receptors. In this way a novel form of selectivity can be obtained based on the unique selectivity features of the binding pockets of the heteromeric complex. The assay systems will then be neuronal cell lines expressing $A_{2A}$ receptors alone and the $A_{2A}/D_2$ heteromeric receptor complex to discover $A_{2A}$ antagonistic drugs with the desired selectivity for the heteromeric $A_{2A}$ coreceptors. Thus, the heteromeric complex is a novel target for drug development (see also Fuxe et al., 1989). It has also been discussed that dimeric compounds can be designed for the heteromeric complex to cointeract with the two binding pockets of the heteromer (Franco et al., 2000; Marshall 2001) to obtain a signaling which better mimic that under physiological conditions. Still another target for drugs could be the interface of the heteromer where drugs can disrupt its formation (see above). Finally, it must be considered that dependent on the heteromeric complex and on the pathological conditions studied it may be bene-

ficial to block or to enhance the intramembrane interactions in the heteromeric coreceptor complex. It must also be emphasized again that in the case of the GABA$_B$ heteromer it has been elegantly indicated that a novel anticonvulsant gabapentin is selective for the GABA R1$\alpha$/GABA$_B$R2 heteromer. Thus, this heteromeric pharmacology has also had an impact on drug development in other types of heteromers (Ng et al., 2001).

- The drug is developed for one coreceptor to address the G protein coupling and G protein selectivity of another coreceptor or the activity of an ion channel receptor existing in the same heteromeric complex. One example is the D$_5$/GABA$_A$ heteromeric receptor complex where activation of the D$_5$ receptor can reduce the synaptic strength of the GABA$_A$ receptor. Thus, activation of the D$_5$ receptor of this complex offers a novel approach for selective reduction of GABA$_A$ signaling in this complex. Thus, GABA$_A$ signaling in discrete brain regions may be reduced in a selective way. It illustrates how drugs can be developed based on various types of heteromeric complexes to reduce or enhance GABA or glutamate synaptic signaling in discrete brain regions. Hence, a novel type of drugs can be developed based on heteromeric complexes containing ion channel receptors and GPCR that may be used to treat a number of neuropsychiatric disorders. It must also be underlined again that the receptor/receptor interaction is reciprocal in the D$_5$/GABA$_A$ heteromeric receptor complex so that the GABA$_A$ receptor activation can control the D$_5$ receptor coupling to its G protein and thus its efficacy. These types of heteromeric complexes may also allow to select drugs preferentially acting on ion channel receptors of heteromeric complexes. It should also be considered that in several examples of heteromerization such as the A$_{2A}$/D$_2$ and A$_{2A}$/mGluR$_5$ receptor heteromers the simultaneous activation of the two binding pockets may also give rise to coupling to other types of intracellular pathways such as the MAPK pathway leading to increased nuclear signaling via transcriptional factor activation with induction of marked changes in gene expression and of the phenotype. In this case development of dimeric agonists for these heteromeric complexes may have a unique and selective trophic potential and help learning and memory processes. It should be considered that the drug developed for one coreceptor may modulate both the binding and the G protein properties as well as the traffic of the other coreceptor.
- The drug is developed for one or both coreceptors to control the receptor trafficking of the heteromeric complex. As an example the A$_{2A}$/D$_2$ heteromeric receptor complex can be mentioned, since prolonged A$_{2A}$ or D$_2$ receptor agonist treatment in vitro alone produced coclustering and a certain cointernaliza-

tion and homologous and heterologous down regulation of A$_{2A}$ receptor function in neuroblastoma cells (Hillion et al., 2002). Prolonged combined treatment with agonists for the A$_{2A}$ and D$_2$ coreceptors produced a much stronger cointernalization and codesensitization involving also the D$_2$ receptor. Thus, it seems likely that increased understanding of the joint regulation by agonists of the trafficking of the A$_{2A}$/D$_2$ heteromeric receptor complex will give us a novel understanding of the desensitization and sensitization at the D$_2$ receptor, a key target for treatment of neuropsychiatric diseases. A$_{2A}$ receptor antagonists may therefore also be used in Parkinson's disease because they counteract the internalization and desensitization of D$_2$ like receptors after prolonged L-DOPA and/or D$_2$ receptor agonist treatment in addition to having an antiparkinsonian and neuroprotective activity (Ferré et al., 2001; Morelli and Wardas, 2001). This may be true also for other heteromeric receptor complexes and therefore offers a new way of avoiding desensitization of key receptors in heteromeric complexes after prolonged agonist treatment, namely by developing drugs that act on the coreceptors.

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