SUMMARY OF RESULTS AND DISCUSSION

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Adenosine A_{2A} and dopamine D_2 receptors heteromerization

The antagonistic interaction between adenosine A_{2A} and dopamine D_2 receptors was first demonstrated in striatal membrane preparations with A_{2A} receptors reducing the affinity of D_2 receptors, especially in the high affinity state for its agonists (Ferre et al., 1991). This offered a novel mechanism for the reported antagonistic adenosine/dopamine interactions found in the brain which was speculated to be an intramembrane interaction (Lepiku et al., 1997). 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 (Dasgupta et al., 1996), 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 can undergo the same modulation by A_{2A} receptor activation, at least at the recognition site level.

In the first work presented in this Thesis, we have reported the existence of a high degree of colocalization of D_2 and A_{2A} receptors in neuroblastoma SH-SY5Y cells stably transfected with D_{2L} receptors and containing native A_{2A} receptors as well as in primary cultures of rat striatal neurons. In addition, it is also shown that A_{2A}/D_2 intramembrane receptor/receptor interaction also has an impact on receptor trafficking. 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 by means of immunocytochemistry in combination with confocal image analysis of nonpermeabilized cells.

Coaggregation was followed by cointernalization of A_{2A}/D_2 receptors after prolonged cotreatment of the neuroblastoma cells with A_{2A} and D_2 receptor agonists. This cointernalization of A_{2A}/D_2 receptors could also be directly demonstrated by incubating fluorescent labelled 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 labelled A_{2A}/D_2 receptors to internalize under the influence of the two agonists.

In cultured striatal neurons a high degree of colocalization of A_{2A} and D_2 receptors was also found, and a prolonged exposure to the A_{2A} agonist or the D_2 agonist could induce coaggregates of A_{2A}/D_2 receptors.

In both, cultured striatal neurons and cell lines, the increasing 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 formation of A_{2A}/D_2 receptor coaggregates was 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. However, an interesting finding was that, 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. The observation that $A_{2A}R$ and D_2R functions are simultaneously altered after long exposure to agonists can aid in understanding behavioural findings involving cross-tolerance and cross-sensitization between dopamine agonists and compounds active at adenosine receptors (such as caffeine) (Fenu et al., 2000).

Although it is a prerequisite, the colocalization of two proteins cannot be taken as a demonstration of a molecular interaction. In this work, we have demonstrated the formation of D_2 and A_{2A} receptors heteromeric complexes by means of coimmunoprecipitation experiments in membrane preparations from D_{2L}-transfected SH-SY5Y neuroblastoma cells and from mouse fibroblast Ltk cells stably transfected with D_{2L} receptors and transiently cotransfected with A_{2A} receptors. However, although commonly used to study protein-protein interactions, coimmunoprecipitation of membrane receptors requires their solubilization using detergents, and it may be problematic when considering highly hydrophobic proteins such as GPCRs that could form artifactual aggregates upon incomplete solubilization (Angers et al., 2002).

Nevertheless, the general acceptance of GPCR dimerization has been lastly demonstrated to exist in living cells with the development and utilization of biophysical methods based on light energy transfer, namely FRET and BRET experiments (see Introduction for more details) (Angers et al., 2000).

Resonance energy transfer approaches are based on the nonradiative transfer of energy between the electromagnetic dipoles of an energy donor and acceptor {Hovius, 2000 #463}. There are two prerequisites for these processes: first, the existence of an overlap between the emission and excitation spectra of the donor and acceptor molecules and, second that the donor and acceptor are in close molecular proximity, typically <100 Å. The critical dependence on the molecular nearness between donors and acceptors for energy transfer (the efficiency of transfer decrease with the 6th power of the distance) makes BRET or FRET systems of choice to monitor protein-protein interactions in living cells. This should be contrasted with confocal colocalization approaches that can provide information about regional but not molecular proximity due the low spatial resolution of light microscopy. Indeed, merging of fluorescent markers will be detected for molecules that can be as much apart as the visible light wavelength (4000–7000 Å). BRET and FRET thus offer unique approaches that allow the monitoring of protein oligomerization in living cells without disrupting the natural environment where they occur. During the last years, an increasing number of papers have appeared confirming previous interactions as well as reporting new ones by the use of various FRET and BRET techniques (Boute et al., 2001; Kroeger et al., 2001; Issafras, 2002; Mercier J.F., 2002; Mercier et al., 2002; Ramsay, 2002; Terrillon et al., 2003)

In the second work presented in this thesis, we used these recent biophysical approaches to further characterize the interaction between A_{2A}/D_2

receptors and also to perform a quantitative analysis of it. Therefore, using the fusion constructs consisting of both receptors fused to fluorescent or luminescent proteins in their C-terminus, the interaction between $A_{2A}R$ and D_2R was further characterized by the use of FRET and BRET. This molecular interaction was found to be specific as when other receptors were assayed no significant signal could be detected. Furthermore, we discarded the possibility of a false signal coming from the close proximity between receptors in some membrane domains (i.e. rafts) where energy transfer has been shown to be possible . Disrupting such structures by cholesterol depletion with β-cyclodextrin, no changes were observed in the BRET signal, supporting the heterodimer notion and ruling out the "false" interaction.

One interesting finding in this work was the observation that the BRET signal in $A_{2A}R/D_2R$ expressing cells was not modified by the activation of $A_{2A}R$ or D_2R by their corresponding agonist, and that similar results were obtained when the two agonists were used simultaneously. The incapacity of neither agonist to modulate BRET could not be attributed to signal saturation, since different donor/acceptor ratios remained insensitive to the ligands. At this point one could argue that this lack of modulation is due to the fact that neither receptor is expressed on the cell surface and, therefore, there's no effect of the ligand. This possibility was ruled out by subcellular fractionation experiments that showed that both receptors were mainly expressed on the cell membrane.

The fact that the A_{2A} and D_2 agonists do not change the BRET efficiency between A_{2A}/D_2 heteromers seems contradictory with the agonist-induced coagregation of both receptors described in the first paper of this thesis. The used BRET technique does not distinguish the different compartments of the cell, and then, events that do not imply changes on the number of dimers but rather reorganization or clustering would not be reflected by this approach. In fact, these results suggest that coagregation doesn't lead to a change in the distance of the two fluorophores, since any variation in the BRET signal was detected.

A number of studies have evaluated the effect of agonists on the level of receptor dimers. Among previous energy transfer-based studies on GPCR oligomerization (using either FRET or BRET), a similar insensitivity to the ligand has been observed for the yeast α-factor receptor (Overton, 2002) and for the human δ-opioid receptor (McVey et al., 2001). In contrast, an agonist-dependent enhancement of energy transfer has been found for somatostatin, β2-adrenergic, gonadotropin-releasing hormone, and thyrotropin- releasing hormone receptors (Angers et al., 2000; Rocheville et al., 2000a; Cornea et al., 2001; Kroeger et al., 2001), and an agonist-promoted decrease of energy transfer has recently been reported for the cholecystokinin receptor (Cheng and Miller, 2001). In some instances, these results have been interpreted as increase, lack of change, or decrease in the amount of oligomers. However, other parameters may explain the observed changes in energy transfer. Both BRET and FRET efficacies vary with the sixth power of the distance between the energy donor and acceptor ($E = Ro^6/(Ro^6+R6)$, where E is the energy transfer efficacy, R represents the distance

between the donor and the acceptor, and Ro is Förster distance at which the efficacy is equal to 0.5) and is also sensitive to their dipole orientations. It follows that all changes that would influence the relative orientation between the donor and the acceptor or the distance between them are also susceptible to induce changes in energy transfer. Thus, changes in distance have a dramatic effect around the R0 but can be undetectable for distances significantly smaller than R0 where the transfer efficacy is reaching its maximum (~1). In fact, changes in distance can be assessed accurately only when the distance between donor and acceptor lies between 0.5R0 and 1.5R0. The lack of effect of the ligands on the BRET detected in several studies could therefore indicate that the distance between Rluc and YFP within the homo- and heterodimers is already smaller than 0.5R0 and cannot be accurately detected. In addition, receptor activation by agonists is associated with conformational changes within the transmembrane core of GPCRs (Ghanouni et al., 2001), coupling to G proteins, receptor phosphorylation by specific kinases, and arrestin translocation. The relative distance and the orientation of the energy donor and acceptor may or may not be affected by these events, depending on their position on the receptor and on the specific structural features of each receptor. Therefore, the agonist-promoted modulation of the energy transfer reported for some GPCRs cannot be readily interpreted as a change in receptor oligomerization. The experimental confirmation of the hypotheses above is provided by a recent study on melatonin receptors showing that ligand-promoted BRET enhancement represents specific ligand-induced conformational changes of preexisting receptor oligomers rather than increased increased oligomerization linked to the activation state of the receptors (Ayoub et al., 2002). With these considerations in mind, it becomes easier to explain why ligand-promoted changes in energy transfer [BRET or fluorescence resonance energy transfer (FRET)] are found in some studies but not in others, depending on the receptors considered.

Energy transfer techniques cannot distinguish the exact oligomerization state (dimer, trimer, tetramer, etc.) of the oligomers, and, still, it remains a matter of controversy. Ayoub et al. performed a competition experiment with melatonin receptors and they fitted their data by adapting the dimer, trimer, and tetramer model of energy transfer quenching proposed by Veatch and Stryer (Veatch and Stryer, 1977) in which the oligomeric state of a receptor could be deduced from an equation (Ayoub et al., 2002). For these receptors they deduced that the dimeric state was the constitutive one. However, further results demonstrating the oligomerization state remain to be obtained as new biophysical techniques are being developed.

Receptor dimerization brought about by the association of two monomers, could be mediated either by covalent (disulfide) and/or non-covalent interactions, and could involve associations of the extracellular domains, TM domains and/or C-terminal tail. However, several studies have suggested that a combination of these interactions occur during dimerization. Therefore, to gain insight into the potential $A_{2A}R/D_2R$ heterodimer interfaces, in collaboration with Dr. Fanelli of the University of Modena, rigid-body docking simulations were done between an average

minimized structure of D_2R and nine different average minimized structures of $A_{2A}R$. From those docking simulations two sets of dimers sharing (within each set) similar interdimer interfaces have been obtained, namely population 1 and population 2.

In the most populated set (population 1), helix 5 and/or helix 6 and the N-terminal portion of I3 from D_2R approach helix 4 and the C-terminal portion of the C-tail from the $A_{2A}R$, respectively. Helix 7(D_2R) may also participate together with helix 6 in the contacts with helix 4($A_{2A}R$). These computational results were then in agreement with the results of a set of competition experiments performed using the BRET approach. The simultaneous transfection of the wild type D_2R was able to decrease the BRET signal (at a $BRET_{50}$ ratio) between $A_{2A}R$-Rluc and D_2R-YFP while when using as a competitor a chimeric D_2R where helices 5 and 6, IL3 and EL3 had been swapped by the corresponding sequence from the D_1R, this receptor failed to decrease the BRET signal even at high amounts of competitor cDNA.

For the GPCR family, recent theoretical studies (Gouldson et al., 2000) support the involvement of the 5th and the 6th transmembrane helices in the dimerization interface as well as an important role for the 3rd intracellular loop. But, interestingly, the results obtained in this Thesis also involve the C-terminal tail of a receptor in these contacts. The inspection of the carboxyl terminus of the $A_{2A}R$ revealed the presence of two adjacent aspartic acid residues, which could interact with an Arg-rich region present in the D_2R I3. In our group, in collaboration with Dr.Woods (National Institute of Drug Abuse, NIH, Baltimore), the peptides corresponding to the relevant epitopes (**VLRRRRKRVN** in D_2R and **HELKGVCPEPPGLDDPLAQDGAVGS** in $A_{2A}R$) were shown to interact, forming non-covalent complexes that were detected by mass spectrometry and that were reinforced by *Ab initio* calculations. However, since the two adjacent Asp present in human $A_{2A}R$ are not conserved among species, another putative epitope for interaction was identified surrounding a serine that can be constitutively phosphorylated in $A_{2A}R$. The peptide corresponding to the phosphorylated epitope (**SAQEpSQGNT**) also formed a non-covalent complex with the D_2R epitope. These results obtained by mass spectrometry were confirmed by using different constructs of the receptors in biochemical pull-down assays. Solubilized D_2R was pulled down by a sepharose-bound GST-fusion protein containing the C-terminal domain of the $A_{2A}R$. Also, the interaction between wild type $A_{2A}R$ and the Arg-rich peptide of the D_2R was displaced by the two peptides corresponding to the two different sequences in the C-tail of $A_{2A}R$. In addition, BRET assays confirmed that mutation of Arg residues in the third intracellular loop of D_2R prevents $A_{2A}R$/D_2R heteromerization. These results represent one of the first examples of epitope-epitope electrostatic interaction underlying receptor heteromerization.

Overall, the present and previously reported data implicate that the membrane interactions taking place between $A_{2A}R$ and D_2R via heteromeric complexes might represent a crucial mechanism influencing D_2R-mediated transmission. As A_{2A} and D_2 are two receptors important for the function of basal ganglia, several of its disorders might be related to their heteromerization.

Parkinson's disease (PD) is a common disorder arising from the degeneration of dopaminergic nigrostriatal neurons, and its symptoms are related to abnormal functioning of the basal ganglia (Albin et al., 1989; Chesselet and Delfs, 1996). Two major output pathways form the basal ganglia, the direct striatonigral and indirect striatopallidal pathway, are responsible for smooth and well-coordinated movement (Gerfen, 1992). Dopamine exerts regulatory control on both pathways, mainly via dopamine D_1 receptors on the striatonigral spiny cells and D_2 receptors on the striatopallidal spiny cells (Gerfen et al., 1990), although the specific expression of dopamine receptors by these neurons is probably not absolute (Surmeier et al., 1992).

Recent studies suggest that motor dysfunction in PD arises in part due to reactive alterations in striatal medium spiny neurons. GABAergic efferent neurons activation changes as dopaminergic innervation declines and especially when their denervated dopamine receptors are subjected to the intermittent stimulation associated with standard dopaminomimetic therapy. In both cases, signalling kinases and phosphatases that regulate the phosphorylation state and thus the synaptic efficacy of coexpressed ionotropic glutamatergic receptors become aberrantly activated. Resultant changes in glutamatergic input evidently modify striatal output in ways that compromise motor function (Chase and Oh, 2000).

Based on these considerations, pharmacological agents that inhibit signaling events in spiny neurons produced by the nonphysiologic stimulation of their dopamine receptors might be expected to alleviate resultant motor dysfunction. Recent observations suggest that drugs interacting with striatal adenosine receptors can serve in this capacity; in fact, adenosine $A_{2A}R$ antagonists have been successfully tested in rodent and primate models of PD (Bibbiani et al., 2003) as well as preliminary clinical observations that lead to support this idea.(Bara-Jimenez et al., 2003).

The present observation that $A_{2A}R$ and D_2R form heteromers and the fact that heteromers function is simultaneously altered after long exposure to agonists, can aid in understanding behavioural findings involving cross-tolerance and cross-sensitization between dopamine agonists and compounds active at adenosine receptors (such as caffeine) (Fenu et al., 2000). Together with other recently reported findings, the present results suggest that changes in $A_{2A}R$ function may be involved in the secondary effects observed after chronic intermittent treatment with L-DOPA such as reduced antiparkinsonian activity and involuntary movements or dyskinesia (Zeng et al., 2000). Adenosine is a feedback detector of neuronal activation, in view of the fact that it allows the neuronal network to return into a resting state, it is therefore expected to increase in the striatal extracellular fluid from patients with Parkinson's disease mainly after chronic intermittent L-DOPA treatment and in response to increased striatal glutamate drive (Chase and Oh, 2000). Hence, striatal extracellular levels of adenosine have been found to increase in the MPTP model of Parkinson's disease (Nomoto et al., 2000). Thus, the wearing off of the antiparkinsonian action of L-DOPA treatment may in part be caused by the simultaneous chronic activation of $A_{2A}R$ and D_2R that, according to the present results, may lead to substantial cointernalization of both receptors.

From the results obtained in this work, it therefore seems likely that the recently demonstrated antiparkinsonian actions of A_{2A} receptor antagonists in humans are to a substantial degree caused by blocking the action of endogenous adenosine on A_{2A} receptors of the A_{2A}/D_2 receptor heteromer, leading to enhancement of D_2 receptor signalling (Ferre and Fuxe, 1992). This may permit the reduction of the L-DOPA dose and thus reduces the development of the L-DOPA–induced dyskinesias related to a change in the phenotypic character of striatal GABAergic neurons with overexpression of prodynorphin and glutamic acid decarboxylase (GAD) mRNA levels (Chen et al., 2003). Furthermore, it has been reported that A_{2A} receptor antagonists alone produce antiparkinsonian effects without dyskinesias in parkinsonian monkeys (Grondin et al., 1999).

Carta et al. (Carta et al., 2003) have shown that combined treatment with an A_{2A} receptor antagonist and a low dose of L-DOPA did not produce the possibly deleterious long-term increases in GAD, dynorphin, and enkephalin mRNA levels. By contrast, repeated treatment with a higher dose of L-DOPA alone (which produced the same acute motor stimulant effect as did the combination of L-DOPA plus A_{2A} antagonist) led to a significant increase in striatal GAD, dynorphin, and enkephalin expression. This absence of striatal gene inductions with repeated L-DOPA plus A_{2A} antagonist was correlated with a stable turning response, in contrast to the sensitized turning response that developed after repeated treatment with L-DOPA alone in this hemiparkinsonian model in rats.

It has also been indicated that long-term L-DOPA therapy requires A_{2A} receptors for persistent behavioural sensitization as studied in A2A receptor knockout mice (Fredduzzi et al., 2002). The loss of inhibition of A_{2A} receptor signaling by the reduced D_2 receptor signaling in patients with Parkinson's disease (PD) adds to the parkinsonian symptoms, and thus antiparkinsonian actions of A_{2A} receptor antagonists can be related not only to an enhancement of D_2 receptor signaling but also to the blockade of increased A_{2A} receptor signaling of the hypodopaminergic state (Fuxe et al., 2001).

Data obtained in the first work on A_{2A}/D_2 receptor cotrafficking suggest that increased A_{2A}/D_2 receptor cointernalization in response to long-term L-DOPA therapy, in combination with increased striatal adenosine tone, may contribute to the deterioration of the therapeutic action of L-DOPA. Simply stated, the desensitization may result from a decreased membrane presence of the D_2 receptor.

These considerations, together with the ones that arise from the A2AR-mGluR5 interaction, described recently in our gorup, are reviewed in the paper "Receptor heteromerization in adenosine A_{2A} receptor signaling. Relevance for striatal functionand Parkinson's disease", 2003, *Neurology* 61, S19-S23. which is supplied in **ANNEX 2**.

The importance of the multimeric complexes in the regulation of the dorsal striatopallidal GABA neurons becomes evident in the treatment of PD. However, these complexes also exist in the ventral part of the striatum, which is related with other neuro-psychiatric disorders such as schizophrenia and drug addiction since, for example, blockade of D_2 receptor by an antagonist and A_{2A} stimulation with an

agonist seem to be improve the antipsychotic effects of neuroleptics or, at least, their therapeutic effect on schizophrenia.

The possible implications of $A_{2A}R/D_2R$ interaction in neuro-psychiatric disorders are reviewed in the paper "Gltutamate mGlu$_5$- Adenosine A$_{2A}$- Dopamine D$_2$ receptor interactions in the striatum. Implications for drug therapy in neuro-psychiatric disorders and drug abuse", 2003, *Curr. Med. Chem* 3, 1-26. which is supplied in **ANNEX 2**.

Adenosine A$_{2A}$ receptors homodimerization

In contrast to heteromerization, homomerization of adenosine receptors has not been extensively studied. In addition, an increasing number of receptors have been reported to form homodimers, these include β-adrenergic receptor (Hebert et al., 1996), dopamine D2 (Ng et al., 1996), mGluR5 (Romano et al., 1996), the δ-opioid (Cvejic and Devi, 1997), the muscarinic M3 (Bai et al., 1998), the vasopressin V2 (Terrillon et al., 2003) or the melatonin M2 (Ayoub et al., 2002). In 1995, our group demonstrated the existence of A$_1$R homodimers in brain tissue from different species (Ciruela et al., 1995). But little attention had been paid to other adenosine receptor homodimers.

In the third work presented in this thesis we took advantage of the FRET and BRET techniques to study A$_{2A}$R homodimerization, using a similar procedure than the one used to characterize the A$_{2A}R/D_2R$ interaction. Therefore, a BRET saturation curve in cells cotransfected with a constant amount of the A$_{2A}$R-*Rluc* construct while increasing concentrations of the A$_{2A}$R-YFP plasmid was analyzed. A positive BRET signal for the transfer of energy between A$_{2A}$R-*Rluc* and A$_{2A}$R-YFP was obtained and shown to be specific since it was hyperbolic and the pair A$_{2A}$R-*Rluc*/GABA$_B$R2-YFP led to undetectable BRET signal. However, as stated for the A$_{2A}$/D$_2$ pair, in heterologous expression systems the detection of energy transfer is not sufficient to invoke dimerization as this phenomenon has been show to occur between closely located molecules distributed in specific membrane domains as lipid rafts (Zacharias, 2002). For this reason β-cyclodextrin treatment was performed on cotransfected cells and, as it didn't lead to changes in BRET signal we could therefore confirm that the BRET signal detected previously was not due to receptor accumulation in lipid rafts. More important is the demonstration by Time-Resolved FRET assays and biotynilation experiments that A$_{2A}$ homodimers exist on the cell membrane as functional species.

As what happened with the A$_{2A}R/D_2R$ heterodimers, stimulation of A$_{2A}$R with its agonist CGS21680 did not promote any change at any BRET ratio, which, as discussed previously, suggested that this dimers were constitutively preformed. For some receptors it is general acceptance that they form oligomers as soon as in the endoplasmic reticulum (ER), and that dimerization seems necessary for them to reach de cell surface ((Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998)), in other cases dimerization has been shown to occur in the ER even though its role remains quite unknown (Issafras, 2002; Overton, 2002; Terrillon et al., 2003) and, yet, some reports suggest that for other receptors dimerization

occurs in the cell membrane and is regulated by ligand stimulation (Angers et al., 2000). Considering these hypothesis, it seems therefore possible that $A_{2A}R$ homodimers as well as $A_{2A}R/D_2R$ heterodimers are preformed in the ER as the lack of effect of the ligands would be in agreement with this supposition. However, and despite the BRET and FRET approaches used were informative for all the cell compartments and subcellular fractionation experiments showed that fused-$A_{2A}R$ were expressed on the plasma membrane.

As has been shown in the previous work, the C-terminal tail of the $A_{2A}R$ seems to be involved in $A_{2A}R/D_2R$ heteromerization, however, we found that this domain was not implied in $A_{2A}R$ homodimerization. In fact, when using an $A_{2A}R$-deletion mutant-flag which lacks the C-terminal region of the molecule immunoblots and co-immunoprecipitation assays confirmed the existence of dimers of these receptors. Therefore, the results obtained so far suggest that, almost in part, different interacting domains are involved in the $A_{2A}R$, homo- and heteromerization.

Demonstration of both molecular interactions between $A_{2A}R$ and $A_{2A}R$ to form homodimers and between $A_{2A}R$ and D_2R to form heterodimers leads to the question which of them is the most favoured interaction. BRET experiments performed using cells cotransfected with either $A_{2A}R$-*Rluc* and $A_{2A}R$-YFP or $A_{2A}R$-*Rluc* and D_2R-YFP implies that the homodimerization is more favored than heterodimerization as it results in higher BRET max and lower $BRET_{50}$ ratios. However, although the level of expression of the fluorescent acceptor in the assays was similar for $A_{2A}R$-YFP and D_2R-YFP, these results should be taken with some caution due to intrinsic differences in fluorescence emission for a given amount of $A_{2A}R$-YFP and D_2R-YFP and to differences in the distance between donor and acceptor in the homo- or the heterodimer.

The discovery of homo- and heterodimerization of GPCRs has revealed a new level of complexity governing the GPCR signaling. Oligomerization and ligand-induced rearrangement (aggregation or clustering) of receptors provides new insight into the understanding of the mechanisms underlying the regulation of receptor function. Therefore, the conformational changes that are transferred through direct receptor-receptor or, generally, receptor-protein interactions, constitute the first level of regulation of receptor function as these interactions can modify signaling characteristics of one or both receptors when they are sequentially or simultaneously activated. The second level of regulation is proposed to occur via indirect interactions between different oligomeric receptor complexes, and is modulated by agonist-induced aggregation of such complexes. This regulation would take place through intramembrane lipids and scaffolding proteins, involving conformational changes in a set of molecules in the membrane and the formation of a molecular circuit (Burgueno et al., 2003b). These levels of regulation are widely discussed in the paper "Regulation of heptaspanning-membrane-receptor function by dimerization and clustering", 2003, *TIBS* 28, 238-243, which is supplied in the **ANNEX 2**.

Overall the results obtained in these first three works support the acceptance of functional complexes in cell membranes.These complexes would not only include receptor-receptor interactions, but they would also be formed by

other scaffolding porteins and would represent a possible mechanism of integration of signals.

Adenosine receptors-induced differentiation

In the fourth work presented we investigated the role of adenosine receptors activation in a model of neuronal differentiation.

The A_1 and A_{2A} adenosine receptors subtypes are the ones mostly expressed in the nervous system, while A_1Rs have the highest abundance in the brain, A_{2A}Rs are mainly localized in few regions such as the striatum, the olfactory tubercle and the nucleus accumbens (Fredholm, 2000). Adenosine levels are dynamically regulated, as cells can release adenosine when there is increased metabolic activity. It has been shown that, hypoxia-induced alterations in the enzymes that influence adenosine metabolism and transport also lead to increases in extracellular concentrations of adenosine (Kobayashi et al., 2000). Adenosine is also formed from ATP, which can be released at the synaptic cleft (Braun et al., 1998) and thus act as a neuromodulator by the effector cells in response to increased metabolic demand. In addition, the fact that both A_1 and A_{2A}Rs are expressed prenatally suggests a possible role of these receptors in neuronal differentiation (Haas and Selbach, 2000; Schulte and Fredholm, 2003).

The implication of adenosine receptors in neuronal differentiation has been studied in several cell lines, they have been reported to stimulate or inhibit survival and differentiation in the nervous system depending on the nature of the cell type and its location.

Recently, it has been reported that A_1Rs are able to inhibit NGF-induced neurite outgrowth in PC12 cells (Thevananther et al., 2001). Since these reported effects were observed following the exposure of a differentiating agent, such as NGF, this suggests that the inhibition occurs after neurite induction has already taken place. Furthermore, the possibility can not be ruled out that this response may be due to the overstimulation of these receptors. On the other hand, it has also been described that antisense mediated inactivation of ecto-5'-nucleotidase, the enzyme that converts ATP to adenosine, inhibits neurite outgrowth in PC12 cells which endogenously express A_{2A}R. (Heilbronn et al., 1995; Arslan et al., 1999). Moreover, also in PC12 cells, agonist stimulation of the A_{2A}R rescued the blockage of nerve growth factor (NGF)-induced neurite outgrowth when the NGF-evoked MAPK cascade was suppressed by an MEK inhibitor or by a dominant-negative MAPK mutant, showing an essential role of cAMP-response element-binding protein activation by A_{2A}Rs in this process (Cheng et al., 2002). Recently, Charles et al. (2003), reported that the bacterial nucleoside MDA (N^6-methyldeoxyadenosine) induces neurite outgrowth through A_{2A}Rs in PC12 cells acting in a synergistic manner with NGF via cAMP and MAPK signalling pathways (Charles et al., 2003). Despite all these previous results, the direct stimulation of adenosine receptors by their classical agonists, independent of growth or differentiation factors, and the possible role this may play in the neuronal differentiation process has not been explored.

In the last part of this thesis we examined whether adenosine receptors can influence neuronal development and the signalling underlying these events. We

tested the effects of adenosine A_1 and A_{2A} receptors activation on neurite outgrowth in SH-SY5Y cells and primary cultures of striatal neurons. Using SH-SY5Y cells as a model, we found that activation of adenosine A_1R and $A_{2A}R$ induces neurite outgrowth and that this neuritogenesis could be considered as an early step of differentiation since stimulation of A_1R and $A_{2A}R$ induced both, the expression of TrkB receptor and the same tendency as retinoic acid (classically used as a differentiation agent) to arrest cells in the G_1 phase of the cell cycle, which are classical effects found in neuronal differentiation processes (Kaplan et al., 1993; Encinas et al., 2000).

The intracellular events triggering neurite sprouting are not well established (Da Silva 2003). Some authors suggest that that the MAPK pathway is involved in the BDNF-mediated survival and neuritogenesis in SH-SY5Y neuroblastoma cells, (Encinas et al., 1999) while others postulate a role for this pathway in gene induction for differentiation but not for the neurite outgrowth processes (Olsson and Nanberg, 2001). In addition, it has been demonstrated that the MAPK pathway plays no role in neural progenitor survival or proliferation but, instead, specifically regulates neurogenesis (Menard et al., 2002; Barnabe-Heider and Miller, 2003). However, in all the previous reports, the importance of the ERK-1/2 phosphorilation is emphasized in the signaling transduction necessary for neuronal differentiation.

In several cell lines it has been demonstrated that all adenosine receptors mediate ERK-1/2 phosphorylation ((Seidel et al., 1999; Schulte and Fredholm, 2003). From our study, in SH-SY5Y cells, both A_1R and $A_{2A}R$, seem to activate the same MAPK pathway in a Ras-dependent manner. However, while the A_1R activation of this pathway was found to be PKA independent, the $A_{2A}R$ response is mediated by PKA, according to the ability of $A_{2A}R$ agonists, but not A_1R, to increase cAMP levels.

In agreement with previously reported data (Olsson and Nanberg, 2001), our results suggest that ERK-1/2 activation is not the only pathway for the morphological differentiation of these SH-SY5Y neuroblastoma cells. In fact, we found that PKC activation is also necessary to induce a full neuritogenesis after stimulation of A_1R or $A_{2A}R$. However, as inhibitors of MEK or PKC are only able to totally prevent neuritogenesis when used in combination, while only partially preventing it when used separately, it is then suggested that MAPK and PKC would be two independent pathways which lead to similar differentiation events. This would be the case of R-PIA, however, a more complex picture occurs with A_{2A} activation. Since inhibitors of PKA activity resulted in a total inhibition of neuritogenesis induced by CGS21680, it is then postulated that in the A_{2A}-mediated differentiation events, PKA activates the MEK/ERK pathway and another pathway that in combination with PKC-dependent pathway results in neurite outgrowth. A not yet identified signaling molecule could act as a link between PKA and PKC or, eventually, a PKA-mediated activation of a component of the signaling cascade could be essential for the PKC translocation to the nucleus and couple this molecule to differentiation effects. Some parts of the exact mechanism of this signaling pathway remains still quite elusive but from our results we can suggest some stages of it which are represented in the scheme shown in Figure 1.

Adenosine is apparently involved in many functions with consequences in the pathology of the nervous system. In fact, adenosine receptors have been suggested to play a role in regulation of sleep (Antle et al., 2001), anxiety (Johansson et al., 2001), cognition and memory (Fredholm, 2000), neuroprotection (Ribeiro et al., 2003), Alzheimer's disease (Maia and de Mendonca, 2002), Parkinson's disease (Schwarzschild et al., 2002), Huntington disease (Reggio et al., 1999), schizophrenia (Ferre, 1997), epilepsy (Dunwiddie T.V., 2001), neuronal maturation (Rivkees et al., 2001) and drug addiction (Knapp et al., 2001). Recently, it has been described that adenosine, acting through adenosine receptors, appears to be the active axon-glial signaling molecule in the CNS, where it promotes oligodendrocyte progenitor cells differentiation into myelitaning oligodendrocytes (Stevens et al., 2002). Impairment of neurite outgrowth has been associated to Alzheimer disease, (Furukawa et al., 1998; Dowjat et al., 1999) and mutations that affect neuronal differentiation cause multiple diseases in humans (da Silva and Dotti, 2002). All these considerations highlight the importance of understanding the mechanisms behind the early stages of neuronal differentiation and the implication of adenosine receptors in these processes.
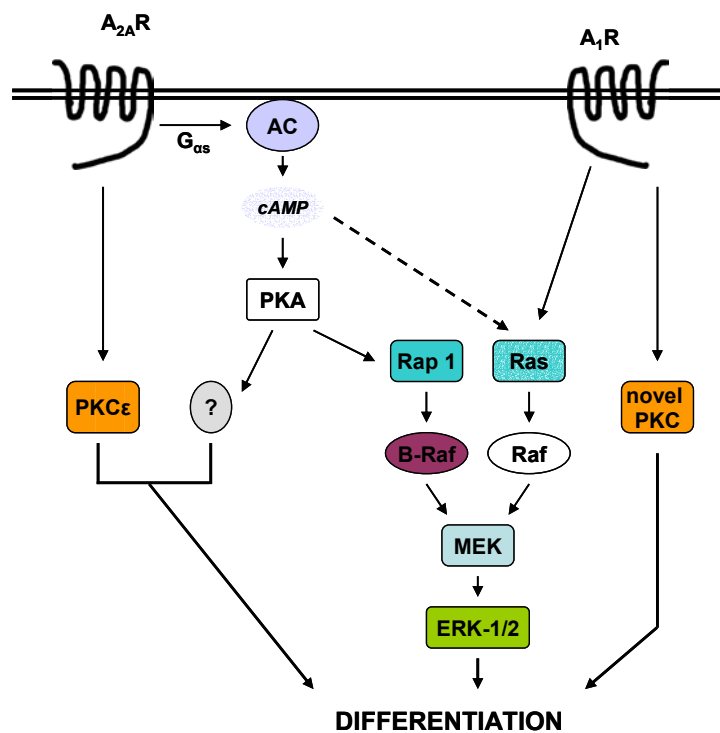

Figure 1: Proposed signalling events triggering differentiation in neuroblastoma SH-SY5Y cells.