THE FUNCTIONAL ASSESSMENT OF FLUORECENTLY TAGGED ADENOSINE A2A AND DOPAMINE D2 RECEPTORS AND QUALITATIVE ANALYSIS OF DIMERIZATION OF ADENOSINE A2A AND DOPAMINE D2 RECEPTOR BY USING FRET

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Submitted by SELİN AKKUZU in partial fulfillment of the requirement for the degree of **Master of Science in Biology Department, Middle East Technical University** by**,**

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Gülay Özcengiz _____
Head of the Department, **Biology**

Assoc. Prof. Dr. Çağdaş D. Son _____
Supervisor, **Biology Dept., METU**

Examining Committee Members

Prof. Dr. Mahinur Akkaya _____
Chemistry Dept., METU

Assoc. Prof. Dr. Çağdaş D. Son _____
Biological Sciences Dept., METU

Assist. Prof. Dr. Hakan Kayır _____
Medical Pharmacology Dept., GATA

Asssoc. Prof. Dr. Mayda Gürsel _____
Biological Sciences Dept., METU

Assist. Prof. Dr. Tülin Yanık _____
Biological Sciences Dept., METU

Date: 28/ 01/2013

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name : Selin AKKUZU

Signature :

ABSTRACT

THE FUNCTIONAL ASSESSMENT OF FLUORECENTLY TAGGED ADENOSINE A2A AND DOPAMINE D2 RECEPTORS AND QUALITATIVE ANALYSIS OF DIMERIZATION OF ADENOSINE A2A AND DOPAMINE D2 RECEPTOR DIMERIZATION BY USING FRET

Akkuzu, Selin

M.Sc., Department of Biology

Supervisor: Assoc. Prof. Dr. Çağdaş D. Son

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Recently, several studies have demonstrated that G protein coupled receptors exist as homo/heterodimers or oligomers. Adenosine A2A receptors and dopamine D2 receptors are present as both homo- and heterodimer. In the GABAergic striatopallidal neurons A2AR are co- localized with D2 receptors (D2R), and establish functional A2AR-D2R heteromers, which modulates dopaminergic activity. Due to be involved in physiological processes, these receptors bear critical roles. Dopamine receptors play critical role in dopaminergic pathways in regulation of memory, food intake and psychomotor activity, etc. On the other hand, adenosine A2A receptors are involved in the regulations of neurotransmission, immune response and cardiovascular systems. Dopamine D2R andadenosine A2AR have been shown to interact in striatum and modulate dopaminergic activity

The purpose of this study is to assess the functionality of EGFP (enhanced green fluorescent protein) and mCherry (a red fluorescent protein) tagged adenosine A2A and dopamine D2 receptors and to detect homo/ hetero-dimerization of these receptors in live cells via Fluorescence Resonance Energy Transfer (FRET). Understanding the mechanisms of the interaction between adenosine and dopamine signaling will help us to figure out some molecular mechanism of neurophysiological disorders. Furthermore, the fluorescence based live cell model could be used to observe the effects of potential anti-psychotic drugs on the interaction of these two receptors.

Keywords: Dopamine D2R, Adenosine A2AR, GPCR Heterodimerization, GPCR Homodimerization, FRET

ÖZ

FLORESAN MOLEKÜLLER İLE İŞARETLENMİŞ ADENOZİN A2A VE DOPAMİN D2 RESEPTÖRLERİNİ İŞLEVSEL TESPİTİ VE RESEPTÖRLERİN EŞLEŞMESİNİN FRET ANALİZİ İLE KANTİTATİF DEĞERLENDİRİLMESİ

Akkuzu, Selin

Yüksek Lisans, Biyoloji Bölümü

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Son zamanlarda yapılan çalışmalar, birçok G proteine kenetli reseptörün (GPKR) homodimer ve heterodimer olarak bulunduklarını ileri sürmektedir. Adenozin A2A ve dopamin D2 receptörleri hem homodimer hem de heterodimer olarak bulunmaktadırlar. A2A receptörleri GABAergic striatopallidal nöronlarında D2 reseptörü ile fonskiyonel A2AR-D2R heteromerini oluştururlar. Bu heteromerlerinj dopaminerjik aktiviteyi düzenlenledikleri tespit edilmiştir. Her iki reseptör de fizyolojik olaylarda kritik roller oynamaktadır. Dopamine reseptörleri, hafızanın düzenlenmesini, besin alımını, ve psikomotor kontrol eden dopaminerjik sistemin anahtar noktasıdır. Öte yandan, Adenozin A2A reseptörünün, sinirsel iletim, kardiyovasküler sistem ve vücudun bağışıklık sisteminde görevleri vardır. Dopamine D2 ve adenozin A2A reseptörlerinin striatumda eşleştikleri ve dopaminerjik aktiviteyi düzenledikleri gösterilmiştir.

Bu çalışmanın amacı; EGFP (geliştirilmiş yeşil floresan protein) ve mCherry (kırmızı floresan protein türevi) floresan proteinleriyle işretlenmiş A2A ve D2 reseptörlerinin işlevsel tespitinin yapılması ve Floresan Rezonans Enerji Transferi (FRET) yöntemine dayanarak aralarında oluşan etkileşimi tespit etmektir. Dopamin-adenozin sinyal yolaklarının etkileşimlerinin mekanizmalarını anlamak için yeni yollar açacaktır. Böylelikle, bazı nörofizyolojik olayların ve bozuklukların da moleküler mekanizmalarının anlaşılmasına katkıda bulunulacaktır. Ayrıca tasarlanan floresan tabanlı canlı hücre modeli, potansiyel anti-psikotik ilaçların bu iki reseptörün eşleşmesi üzerine etkilerinin tespiti için kullanılabilecektir.

Anahtar Kelimeler: Dopamin D2R, Adenozin A2AR, GPKR Heterodimerizasyonu, ve homodimerizasyonu , FRET

To my family

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ABBREVIATIONS

$A_{2A}R$	Adenosine A2A Receptor
AC	Adenylyl Cyclase
ADHD	Attention Deficit Hyperactive Disorder
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
bp	base pair
BiFC	Bimolecular Fluorescence Complementation
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
cAMP	cyclic AMP
CFP	Cyan Fluorescent Protein
CCR5	C-C Chemokine Type 5 Receptor
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CoIP	Co-Immunoprecipitation
D-MEM	Dulbecco's Modified Eagle Medium
D-PBS	Dulbecco's Phosphate Buffered Saline
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
FRET	Fluorescence/Förster Resonance Energy Transfer
G_i	Inhibitory Gα subunit
G_{olf}	Olfactory Gα subunit
G_s	Stimulatory Gα subunit
GABA	γ-amino Butyric Acid
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GIRK	G-protein gated inward rectifying potassium channels
GnRH	Gonadotropin Releasing Hormone
GPCR	G-Protein Coupled Receptor
GTP	Guanosine Triphosphate
IL	Interleukin
kb	Kilobase pair
LB	Luria Bertani
MAP	Mitogen Activated Protein
mCherry	monomericCherry
mGluR	Metabotropic Glutamate Receptor
mRNA	Messenger Ribonucleic Acid
N2a	Neuro2a
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PLC	Phospholipase C
PLD	Phospholipase D
RE	Restriction Enzyme
rpm	Revolution per Minute
Taq	Thermus aquaticus

TBE	Tris Borate EDTA
UV	Ultraviolet
YFP	Yellow Fluorescent Protein

CHAPTER 1

INTRODUCTION

1.1 G-Protein Coupled Receptors

G-Protein Coupled Receptors (GPCRs) belong to the largest integral membrane protein family. It has been reported that about 1000 genes in human genome encode GPCRs (Kobilka, 2007; Takeda, Kadowaki, Haga, & Takaesu, 2002). At structural level, GPCRs demonstrate conserved structural features such as seven trans-membrane alpha helices which are attached by three extracellular and three intracellular loops with extracellular amino (N)-terminus and intracellular carboxy (C)-terminus (Figure 1.1) (Kobilka, 2007). The conserved motifs and ligand-binding site of receptors are often used to define the subfamilies of GPCRs and there have been five main subfamilies, namely: adhesion, frizzled/taste 2, glutamate, rhodopsin and secretin (Fredriksson, Lagerström, Lundin, & Schiöth, 2003; Parrill & Bautista, 2010). GPCRs have been involved in different signaling processes in response to hormones and neurotransmitter. Besides, they have been involved in sensory stimulation processes, especially induced by light and odorants (Rohrer & Kobilka, 1998). Additionally, cellular processes including inflammation, immune response, cellular differentiation and cell growth are mediated by GPCRs (Bouvier, 2001).

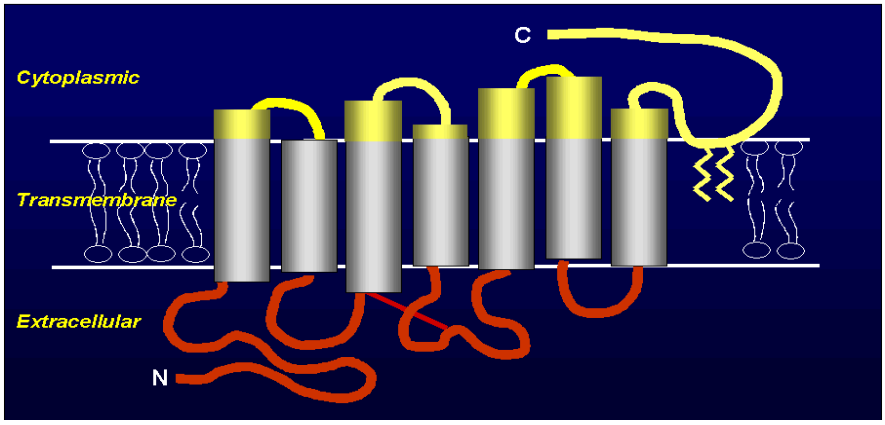

Figure 1.1 The representative image of GPCR – seven transmembrane structures divides the protein into three domains, the cytoplasmic, the transmembrane and the extracellular domains. (Taken from (© 2002 Judith Klein-Seetharaman, University of Pittsburgh)

As a result of ligand binding to GPCRs, receptors change conformation and the active form of receptor will be stabilized so that the GDP bound to the α subunit will be replaced by GTP, thus heteromeric G protein (GTP –binding proteins), which is composed of 3 subunits, namely α, β and γ, will be separated

from the GPCR and dissociates from each other. When ligand molecules bind to GPCRs, inactive state of Gα protein, which is bound to GDP, becomes active by replacement of the GDP with GTP; thereby conformational change in G protein occurs and Gα bounded to GTP dissociates from receptor and Gβγ, Gβγ dissociate from the receptor but stay as a hetero dimer (Lambright *et al*., 1996). Dissociated heterotrimeric subunits of G proteins can initiate different signaling cascades. Different subtypes of Gα exert their effects on different effectors such as ion channels, adenylyl cyclase, and phospholipase C (Neer, 1995) (Fig.1.2). The receptor-mediated activation either inhibits or stimulates these effectors to change concentrations of molecules such as calcium and cAMP as secondary messengers (Strader, Fong, Tota, Underwood & Dixon, 1994). Similarly, Gβγ dimer effectors including PLC2 and 3, phosphoinositide 3-kinase (PI3 kinase), potassium and calcium channels and importantly, components of the MAP kinase cascade are regulated by receptor mediated G protein activation (Cabrera-Vera, 2003) (Fig 1.2).

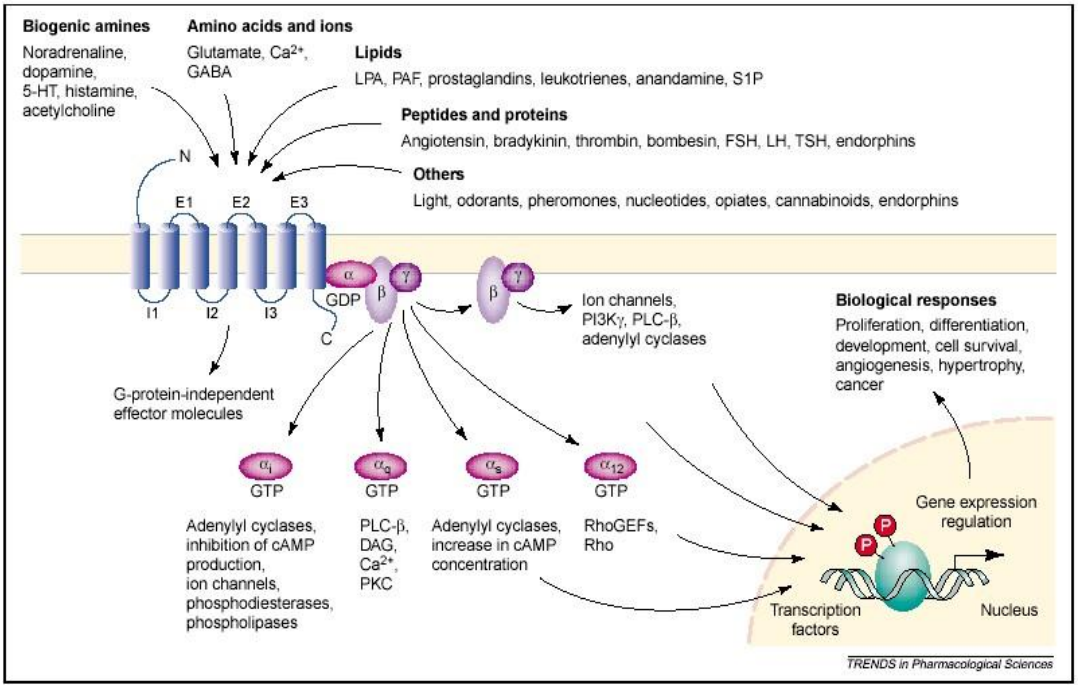

Figure 1.2 GPCR signaling upon a diverse range of ligand binding and the downstream of several GPCR pathways (taken from (Dorsam & Gutkind, 2007))

Having critical functions in human physiology makes GPCRs the drug targets of about 25% of total marketed pharmaceuticals (Haack & McCarty, 2011) . These drugs have been designed considering the modulations of GPCRs such as G-protein interactions and blocking or activating receptor induced signaling pathways (Eglen & Reisine, 2011). In the market, mimetic of agonist and antagonist of Drugs targeting GPCRs have been used against several disorders such as hypertension, allergies, asthma, psychotic disorders and migraine (Bouvier, 2001). Many next generation drugs like allosteric regulators have been produced with novel pharmacology also target these receptors (Eglen & Reisine, 2011).

1.1.1 Dopamine Signaling

Dopamine is a catecholamine derivative neurotransmitter (Fig 1.3). It acts as a neuromodulator that is used by neurons in different regions of the brain, and involves in many neural processes such as motor function, cognition, learning, and emotion. Effects of Dopamine on neurons occur through dopamine receptors, which are also members of G-protein coupled receptor superfamily. Dopamine receptors have been classified into five subtypes, D1, D2, D3, D4 and D5 within two subfamilies. However, properties of signal transduction and signaling pathway at molecular level determine the classes of dopamine receptors as D1- like and D2- like dopamine receptors (Neve, Seamans, & Trantham-Davidson, 2004). According to this classification, D1 and D5 types of dopamine receptors are involved in the D1-like dopamine receptor subfamily, on the other hand, other three dopamine receptors, D2, D3 and D5 belongs to the D2–like dopamine receptor subfamily (Gingrich & Caron, 1993).

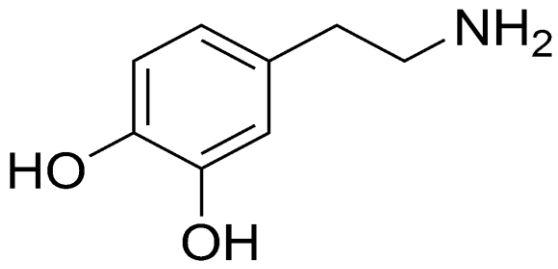

Figure 1.3 Dopamine structure (see Appendix A for metabolic reactions)

All of the dopamine receptors are members of GPCRs so the signaling pathway of dopamine receptors is mediated by activation of heteromeric G proteins resulting from the interaction with receptors (Neve *et al.*, 2004). Although subfamilies of dopamine receptors share the common structures of 7- transmembrane receptors, there are structural differences between D1-like and D2-like dopamine receptor subfamilies. At structural level, D1–like receptors have a longer C–terminal tail and a shorter 3rd intracellular loops differing from D2–like receptors. Because of having potential phosphorylation sites at the 3rd intracellular loop of dopamine receptor, this loop is inclined to interact with G protein in both subfamilies of dopamine receptors (Missale, Nash, Robinson, Jaber, & Caron, 1998). Besides, the structural variants, D1-like and D2-like dopamine receptors show differences in the signal transduction pathways. The signal transduction of dopamine receptors can initiate adenylyl cyclase (AC), calcium channels or potassium channels. Subfamilies of dopamine receptors show inverse effect on AC activation in terms of cAMP production. The activation of D1–like receptors upon agonist binding results in the accumulation of cAMP whereas the activation of D2-like receptors inhibits the AC activity on production of cAMP. Being coupled to stimulatory $G\alpha_s$, D1-like dopamine receptors mediate the activation of AC, which catalyzes the conversion of ATP to cAMP. As a result of binding of cAMP to regulatory subunits of protein kinase A (PKA) holoenzyme the catalytic domain will get activated. Thus, PKA which is involved in the phosphorylation of many proteins in the cellular metabolism becomes active (Neve *et al*., 2004). Unlike D1-like receptors, D2-like receptors are coupled to inhibitory $G\alpha_i$ subunits. Since $G\alpha_i$ binds to AC to inhibit enzyme activity

resulting in the decreased cAMP level, D2-like receptors reduce PKA -stimulated phosphorylation (Neve *et al.*, 2004).

As a neuromodulator, dopamine affects fundamental processes in mammals. Mainly, neural processes such as reward, learning, motor control, emotion are mediated by dopamine–induced signaling pathways (Gingrich & Caron, 1993). Because of the importance of dopamine in neural processes, the dsyregulation of the dopamine–induced signaling can be correlated with many pathological disorders such as Parkinson's disease, schizophrenia, Tourette's syndrome, and hyperprolactinemia. The pharmacological profile of dopamine receptors, in terms of agonists and antagonists are used as a model for drug targeting against these diseases. To illustrate, as a reducing agent of hypokineasia in Parkinson's diseases, and as a blocking agent of symptoms of schizophrenia such as hallucinations and delusions, agonists and antagonists of dopamine receptors have been designed, respectively (Missale *et al.*, 1998). Therefore, in order to manage these conditions, drugs targeting dopaminergic mechanisms have been developed considering the pharmacological profiles of dopamine receptors including agonists and antagonists (Yao Wei-Dong, Spealman Roger D, 2008). The pharmacological profiles of dopamine receptors are summarized as shown in Table 1.1 (Missale *et al.*, 1998).

Table 1.1 Pharmacological properties of dopamine receptors (ND: not determined; ++++: 0.5 nM < Ki < 5 nM ; ++ : 5 nM < K i < 50 nM ; + : 50 nM < Ki < 500 nM ; +/- : 500 nM < Ki < 5uM; - : Ki > 5 uM) (adapted from (Missale *et al.*, 1998))

	D1- like		D2 –like		
	D1	D5	D2	D3	D4
Antagonists					
Clozapine	+	+	+	+	++
(+)-Butaclamol	+++	++	+++	ND	++
Chlorpromazine	+	+	+++	+	++
Agonists					
Dopamine	+/-	+	+	++	++
Bromocriptine	+	+	+++	+++	+
Qinpirole	-	ND	+/-	++	++

1.1.1.1 Dopamine D_2 Receptors

The dopamine D2 receptors belong to the D2-like subfamilies of dopamine receptors, which are members of G-protein coupled receptor superfamily. Like all GPCRs, dopamine D2 receptors have a common structure including seven transmembrane segments. However, the 3rd intracellular loop of receptors shows variations among D2 receptors. Because of this variation, there have been two isoforms of dopamine D2 receptors, long isoform (D_{2l}) and short isoform (D_{2s}) mediated by alternative splicing of transcript. This structural variance results from the insertion of 29 amino acids in 3rd cytoplasmic loop of

long isoform of D2 receptors (D_{2l}) (Dal Toso *et al*., 1989). This loop seems to be involved in the coupling of receptor with heteromeric G-proteins (Prou *et al*., 2001). Despite the insertion on this critical location, which is involved in the interaction with $G\alpha_i$ functional difference between two isoforms has not been detected (Missale *et al*., 1998). Both isoforms of dopamine D2 receptors are coupled to Gαi/olf proteins, which mediates the D2 dopamine receptor inhibition of adenylate cyclase (Prou *et al*., 2001). Therefore, the stimulation of dopamine D2 receptors reduces the PKA–stimulated phosphorylation of proteins (Neve *et al*., 2004). Both isoforms exert the same effect on signaling pathway when they are expressed in the cell line; however, in terms of the binding affinity of isoforms to G protein, D_{2s} isoforms have higher affinity than D_{2l}(Dal Toso *et al*., 1989). Besides the difference of binding affinity, the amount of D2 dopamine isoforms shows variation among different brain locations (Giros *et al*., 1989).

In order to understand molecular processes, which mediate dopaminergic effect, the mechanisms involving the coupling of receptor with $G_{\alpha i/olf}$ proteins are the most studied modulations. However, there are other modulations, which are associated with D2R gene (drd2). The studies about alcoholism, co-morbid depression, anxiety and social dysfunction in untreated veterans with post-traumatic stress disorder show that drd2 gene is involved in the signaling cascades of these diseases (Lawford, Young, Noble, Kann, & Ritchie, 2006; Thanos *et al*., 2005). As a result of dopaminergic regulation, the activation of Akt signaling/glycogen synthase kinase 3 (GSK-3) is induced by dopamine D2 receptors (Beaulieu & Gainetdinov, 2011). The activation of D2 receptors is involved not only in Akt signaling but also in Wnt signaling pathway (Min *et al*., 2011). The inhibition of Wnt pathway via interaction with β-catenin induced by D2 receptor activation affect the cytosolic level of β-catenin as a transcriptional co-activator for transcription factor LEF/TCF (lymphoid enhancing factor/T-cell factor) in the nucleus (Cadigan & Liu, 2006). In addition to Akt and Wnt signaling cascades, the activation of the D1-D2 heteromer is linked to signaling cascade associated with intracellular calcium release (George & O'Dowd, 2007).

Besides the brain areas, dopamine D2 receptors are also expressed, in cardiac tissue. The activation of this receptor upon agonist binding in cardiac tissue causes the decrease in heart rate and arterial blood pressure (Végh, Papp, Semeraro, Fatehi-Hasanabad, & Parratt, 1998). Agonists of dopamine D2 receptors can be new therapeutic agents for myocardial ischemia/reperfusion injury inducing cardiomyocyte apoptosis (Li *et al*., 2011).

1.1.2 Adenosine Signaling

In the central and peripheral nervous system, adenosine, a purinergic messenger, serves as neuromodulator involved in the neuronal and non- neuronal cellular processes (Sawynok & Liu, 2003) (Fig.1.4). The adenosine molecule exerts its effect through the adenosine receptors including four subtypes, A1, A2A, A2B and A3, which are members of the GPCR superfamily (Olah, Ren, & Stiles, 1995). Like other GPCRs, adenosine receptor subtypes are coupled to heteromeric G-proteins mediating a variety of transduction mechanisms. In the brain, the A1 receptors, which are highly abundant, can be coupled to stimulatory and inhibitory Gα subunits. The coupling with stimulatory Gα, also known as Gs, results in activation of potassium (K^+) channels (Trussell & Jackson, 1985); on the other hand interaction with inhibitory Gα, known as Gi, inhibits the adenylyl cyclase activity leading a decrease in cAMP level. Moreover, it inhibits presynaptic calcium (Ca^{+2}) channels (MacDonald, Skerritt, & Werz, 1986). Therefore, the K^+ channel activation and Ca^{+2} channel inhibition lead to inhibition of neuronal activity (Ribeiro, Sebastião, & De Mendonça, 2002). A2 adenosine receptors, which are primarily expressed in the striatum (Fredholm, Chen, Masino, & Vaugeois, 2005), are linked to stimulation of adenylyl cyclase: thereby increase the cAMP level. In addition to brain areas, adenosine A2 receptor are also expressed in

blood vessels so they exert their effects as a vasodilator effect in the brain (Shi *et al*., 2007). Unlike A2 receptor, A2B receptors are expressed at different regions of brain excluding striatum, where they are expressed at medium or low abundance, and also A2B receptors are coupled to Gs; therefore, adenylyl cyclase and phospholipase C (PLC) are activated. PLC is also activated by A3 adenosine receptor. Additionally, A3 receptor is involved in regulation of intracellular Ca^{+2} level by increasing Ca^{+2} amount (Dunwiddie & Masino, 2001). As a result of activation of all subtypes of adenosine receptors, adenosine signaling is involved in several metabolic processes including modulation of excitability in the central nervous systems (CNS), induction of sleep, perception of pain etc. (Benarroch, 2008). Because of the involvement in serious processes in living organisms, agonists and antagonists of adenosine receptors are thought to be models for drug targeting in several diseases such as cerebral ischemia, pain, seizures and Parkinson's diseases (During, Ryder, & Spencer, 1995; Ribeiro et al., 2002; Sawynok & Liu, 2003; Schapira *et al*., 2006).

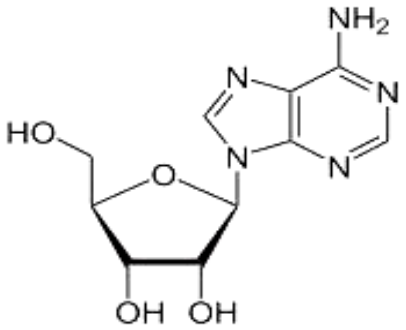

Figure 1.4 Adenosine structure (see Appendix A for metabolic reactions)

Another regulator affecting adenosine signaling is adenosine concentration. Adenosine transporters, intracellular and extracellular enzymes are contributed into adenosine signaling under some conditions. To illustrate, stress conditions trigger the formation of adenosine monophosphate (AMP), as a result of dephosphorylation of adenosine triphosphate (ATP), and then, AMP is converted to adenosine by the enzyme, 5` nucleotidase. Intracellular and extracellular adenosine molecules are transported by nucleoside transporters such as equilibrative nucleoside transporters (ENT) for membrane translocation (King, Ackley, Cass, Young, & Baldwin, 2006). Intracellular and extracellular adenosine concentration can be fluctuated under abnormal conditions such as hypoxia, trauma, seizures etc. (Ribeiro *et al*., 2002). The biological properties of types of adenosine receptors are summarized in Table 1.2.

Table 1.2 Biological characteristics of Adenosine Receptor subtypes (adapted from (Xu, Bastia, & Schwarzschild, 2005)

	A1	A2A	A2B	A3
G-protein	Gi1/2/3, G0	Gs, Golf, G15/16	Gs, Gq/11	Gi2,3, Gq/11
Signaling pathway	cAMP decrease IP3/DAG increase	cAMP increase IP3 increase	cAMP increase IP3/DAG increase	cAMP decrease IP3/DAG increase
Distribution In brain	(Highly expressed in cortex, cerebellum and hippocampus;	Highly expressed in striatum, nucleus accumbens, olfactory tubercle	Low levels in all areas)	Intermediate levels in cerebellum and hippocampus
Receptor Binding Affinities (Ki, in nM)				
A2A agonists				
Adenosine	70	150	5100	6500
NECA	14	20	330	6.2
CGS21680	290	27	361000	67
A2A antagonists				
Caffeine	12000	2400	13000	80000
MSX-2	2500	5.0	-	>10000
SCH58261	290	0.6	-	>10000

1.1.2.1 Adenosine A_{2A} Receptor

Adenosine A2A receptor is a member of G- protein coupled receptors. When it is activated upon ligand binding, it is coupled to G_s or G_{olf} leading to the activation of AC and therefore, increasing cAMP concentration (Fredholm *et al*., 2005). Moreover, the binding affinity of A2 receptor to adenosine is higher than other subtypes of adenosine receptors including A1, A2b and A3 (Daly, 1982). At the structure level, A2 receptor whose molecular weight is 45 kDa is a glycoprotein, which consists of a single carbohydrate chain. Moreover, it has a consensus site for N- linked glycosylation (Olah *et al*., 1995; Xu *et al*., 2005). Different from other adenosine receptor subtypes; adenosine A2 receptor has an insertion of 80-90 amino acids at the carboxy-terminal tail (Olah *et al*., 1995). The localization of this receptor subtype in the brain regions is abundantly in striatum, and also in cortex and midbrain with less expression (Fink *et al*., 1992). In addition to brain areas, the expression of A2 receptor is detected in heart, blood vessels and lung at intermediate levels (Shi *et al*., 2009).

Recent studies indicate that A2 adenosine receptors are also expressed on glial cells (Popoli *et al*., 2007). As a result of receptor activity in this area, it leads to myelination in some cells such as Schwann cells (Stevens *et al*., 2002). The study shows that A2 receptor upon antagonist interaction can be critical for transition astrocytic response when exposed to some situations such as injury and inflammation. According to Brambilla *et al*., selective antagonist of A2 receptor, SCH58261, is optional preventing agent for growth factor induced elongation in cellular mechanisms such as astrogliosis (Brambilla, Cottini, Fumagalli, Ceruti, & Abbracchio, 2003). It is shown that activation of receptors has neuroprotective effect on meningitis (Sullivan, Linden, Buster, & Scheld, 1999); on the other hand, its activation induces ischemic brain injury when they are expressed on bone marrow-derived cells (Yu *et al*., 2004).

1.2 Interaction of Adenosine A2A and Dopamine D2 receptors

Adenosine A2 and dopamine D2 receptors, which are abundantly expressed in striatopallidal GABA (γ-aminobutyric acid) containing neurons, exert the antagonistic interaction, in terms of signal transmission (Ferré *et al*., 2004; Soriano *et al*., 2009). Being functionally involved in pathophysiology of disorders located in basal ganglia makes these neurons key point in Parkinson's disease, drug abuse, as well as neuroleptics (Canals, Marcellino, *et al*., 2003). Therefore, this antagonistic interaction is thought to be a base in order to develop therapeutic approaches against neuropsychiatric diseases such as schizophrenia and Parkinson's disease (Ferré *et al*., 2004).

The interaction between adenosine A2A and dopamine D2 receptors have been demonstrated at biochemical, behavioral and functional levels. At the biochemical level, this antagonistic interaction can be observed in two outcomes. First one is the stimulation of adenosine A2 receptor upon ligand binding has antagonistic effect on dopamine D2 receptor so binding affinity of agonist to dopamine D2 receptor decreases upon stimulation of A2A receptor. (Björklund *et al*., 1997). Second is that the interaction of adenosine A2 and dopamine D2 receptor in the intramembrane exerts its effect through $G_{s/olf}$- and G_i-coupling on adenylyl cyclase activity (Ferré *et al*., 2004). As mentioned before, Adenosine A2 receptor is coupled to $G_{s/olf}$ to stimulate the AC activation, which is inhibited by dopamine D2 receptor activation, as a result of G_i- coupling. Therefore, the cAMP level is affected by this antagonistic interaction, which leads to contradicting changes in cellular processes such as protein phosphorylation and gene expression (Hillion *et al*., 2002).

1.3 Dimerization of G-Protein Coupled Receptors

A novel concept of GPCR dimerization or oligomerization contradicts the classical idea about the existence and functioning of GPCR as monomeric species (Milligan, 2004). It is shown that, functionally GPCR can exist as dimers, not only with themselves to form homo-dimers but also with closely or distantly related other GPCR members to form hetero -dimers (Rios, Jordan, Gomes, & Devi, 2001). The studies about GPCR dimerization have indicated that dimers form early, after biosynthesis, and dimerization is thought to be involved in receptor maturation. Additionally, dimerization of receptors have influenced some processes including G-protein coupling, regulatory processes, and downstream/upstream signaling (Terrillon & Bouvier, 2004). As the initial proof for dimerization, Liang *et al* showed that the rhodopsin exist as homo -dimer, as a result of atomic force microscopy (AFM) studies. Moreover, assays

deriving from AFM applications demonstrated the oligomeric organization of rhodopsin (Liang *et al.*, 2003). Similarly, co-immunoprecipitation, bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) studies have been used to visualize or detect the dimerization of GPCRs. GABAB receptors are evidence that GPCR-GPCR interaction begins during biosynthesis and can be important for the function of GPCRs (Milligan, 2004). Marshall and her colleagues demonstrated that GABAB receptors form heterodimer (Marshall, Jones, & Kaupmann, 1999); in order to properly traffic to plasma membrane, GABAB2 subtypes of receptor need GABAB1 subtypes (Villemure *et al.*, 2005).

Baneres and his colleagues showed that leukotriene B4 (BLT1 receptor) dimers are engaged with only one heteromeric G-protein after an agonist treatment. By solution phase neutron-scattering experiment, they proved that GPCRs could exist as a dimer, supporting 2:1 stoichiometry of GPCR: heteromeric bounded G-protein. Another study about metabotropic glutamate (mGLU) receptors supports the dimerization of GPCRs, in terms of the operative unit. According to this study, the stimulation with an agonist induces not only the partial activation of dimers, but also optimal coupling of dimer with G- proteins, as a result of the activation of double promoter (Kniazeff *et al.*, 2004). Therefore, it can be concluded that GPCRs are functionally inclined to be dimer. Moreover, GPCRs become dimer in the process of biosynthesis, trafficking and maturation. Many studies including GABAB receptors indicate that the beginning of the dimerization is early in the biosynthesis, which occurs in the ER. Dimerization in the ER is constitutive; differ from agonist-induced dimerization (Bulenger, Marullo, & Bouvier, 2005). Like GABAB receptors, α1D- and α1B–adreno-receptors need to be heterodimerize in ER to be properly expressed at the cell surface (Hague, Uberti, Chen, Hall, & Minneman, 2004). Furthermore, β2–adrenergic and related olfactory receptors form hetero-dimers, which is enough to moderate the trafficking of the dimer to cell membrane (Hague, Uberti, Chen, Bush, *et al.*, 2004). Lastly, hetero-dimerization of taste receptors such as T1R2 and T1R3 is necessary for the biogenesis of taste receptors (Nelson *et al.*, 2002).

Internalization of GPCR might also be influenced by hetero-dimerization, in terms of agonist–induced GPCR endocytosis(Terrillon & Bouvier, 2004). Several studies demonstrate that induction of only one of the promoters was enough to lead co-internalization of both receptors in the dimer. δ-opioid /β2 adrenergic (B. a Jordan, Trapaidze, Gomes, Nivarthi, & Devi, 2001) , SSTR1/SSTR5 somatostatin (Rocheville *et al.*, 2000) , A2A adenosine/D2 dopamine (Hillion *et al.*, 2002), α2A/β1 adrenergic (Xu *et al.*, 2005), SSTR2A somatostatin/ μ opioid (Pfeiffer *et al.*, 2002) and α1A/α1b adrenergic (Novi *et al.*, 2005) receptors are all capable to be co-internalized. Furthermore, cross desensitization in the signaling pathway promote co-internalization of receptors. Some studies have shown the opposite mechanism, that is hetero-dimerization of some GPCRs demonstrate negative effect on agonist induced endocytosis (Terrillon & Bouvier, 2004). To illustrate, the β1AR prevent the agonist-mediated internalization of the β2AR (Lavoie *et al.*, 2002): similarly, the κ-opioid receptor has inhibitory effect on the endocytosis of both β2AR (B. a Jordan *et al.*, 2001) and δ-opioid receptors (B. A. Jordan & Devi, 1999).

1.3.1 Methods to Detect Dimerization of G-Protein Coupled Receptors

The experimental approaches including biophysical, biochemical and functional complementation assays provide the direct or indirect evidence for existence of G-protein coupled receptor dimerization. Additionally, these techniques have been used to investigate both pharmacological and functional properties of GPCRs (Milligan & Bouvier, 2005). Firstly, as biochemical approach, co-

immunoprecipitation (CoIP) has been used to confirm the oligomerization of GPCRs. This method is based on the ability of an antibody to stably and specifically binding complexes containing a bait protein that interacts with other proteins as homo/hetero-dimer form. As a type of co–immunopreicipation, differentially epitope tagged CoIP have been preferred to be used for detection of dimerization of GPCRs, especially calcium sensing receptors (Ward, Brown, & Harris, 1998) and histamine H2 receptors (Fukushima *et al.*, 1997). First application of CoIP by Hebert and colleagues was performed to detect receptor dimerization of β2–adrenergic receptors, which were tagged with hemagglutinin (HA) – and c-myc tags and were co-expressed in insect Sf9 cells. Immunoprecipitation with the anti-HA and anti c-myc antibody including dimer of receptors were obtained. In order to detect specific interaction, muscarinic Ach receptor M2 was tagged with c-myc tags, instead of c- myc tagged β2–adrenergic receptor and it was expected not to see co-immunoprecipitation between two receptors (Hebert *et al*., 1996). Beside homo-dimerization, hetero-dimerization of many GPCRs including dopamine D1 (Ginés *et al*., 2000), adenosine A1, bradykinin B2 and angiotensin I receptors (AbdAlla, Lother, & Quitterer, 2000) have been demonstrated. Although co-immunoprecipitation is mainly preferred to show the GPCR dimerization, there are some problems with this technique. Major problem or concern in the application of CoIP is false positives due to aggregation since GPCRs have hydrophobic nature (Rios *et al*., 2001). In the solubilization and immunoprecipitation steps, some reagents such as reducing agents are used to reduce artifactual aggregation of GPCRs; however, using reducing agents sometimes cause a decrease in level of dimer formation (B. A. Jordan & Devi, 1999). In addition to reducing agent, a capping agent affecting cysteine residues can be used to decrease artifactual aggregation, as a result of formation of disulfide bond in a receptor (K. Kroeger, Pfleger, & Eidne, 2005). Secondly, biolimunescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) methods, as biophysical approaches, have been used to visualize dimerization of GPCRs in living cells. In BRET method, the energy transfer from emission of light resulting from degradation of coelenterazine in luciferase to fluorescent protein such as green fluorescent protein (GFP) is being used to detect the interaction (Milligan, 2004). Angers and his colleagues carried out first BRET study with β2-adrenergic receptors (Angers, Salahpour, & Bouvier, 2002). In this study, β2-adrenergic receptors were genetically linked with Renilla luciferase (Rluc) and GFP proteins and then energy transfer between these fluorescent tagged receptors, which were transfected into same cells, was detected, as a result of constitutive homo-dimerization of β2 -adrenergic receptors (Angers *et al*., 2002). However, there were some limitations in this study. One of these limitations is that low sensitivity of this method is not favorable for subcellular localization, in terms of dimerization of GPCRs. To overcome the problem, low sensitivity, more sensitive cameras and/ or luminescence donors which can give higher light output can be used (Milligan & Bouvier, 2005). BRET method was used to visualize dimerization of other GPCRs such as δ opioid receptors (McVey *et al*., 2001) and thyrotropin-releasing hormone receptors (K. M. Kroeger, Hanyaloglu, Seeber, Miles, & Eidne, 2001). In addition to homo-dimerization, hetero-dimerization can be detected by BRET method. Similar to BRET, fluorescence resonance energy transfer (FRET) technique has been carried out by using donor molecules and acceptor molecules. Different from BRET, external light source is used to excite donor molecule, which is a variant of fluorescent protein in most cases. If the distance between two fluorescent protein tagged proteins is less than 100 Å, the energy from excited donor molecule can be transferred to acceptor molecule and the acceptor molecule can be excited (Clegg, 1995). Therefore, FRET methods allow direct access to obtain the information of the interactions of two proteins in living cells in nanometer scale. For instance, Overton and his collogues visualized the oligomer structure of the GPCR that is α-factor receptor, the product of the STE2 gene in the yeast Saccharomyces cerevisiae (Overton & Blumer, 2000). In addition to oligomerization of Ste2p, the dimerization of gonadotropin releasing hormone (GnRH) receptor, which is mediated upon agonist binding, was detected using FRET method with GFP and red fluorescent protein (RFP) (Cornea, Janovick, Maya-Núñez, & Conn, 2001). Moreover, luteinizing hormone (LH) receptor dimerization was shown by FRET via labeling hormone with two different flourophores (Roess & Smith, 2003). In addition to biophysical and biochemical assays, functional complementation assay is another technique to detect dimerization of GPCRs. This method based on direct protein-protein interaction has two receptors, which are nonfunctional GPCR mutants that are capable to reconstitute agonist -mediated

functional receptor (Monnot *et al.*, 1996). First application included two nonfunctional α2 -adenergic receptors with mutations in transmembrane (TM) 1 and 5 and M3 muscarinic receptor with mutations with TM 6 and 7. When expressed alone neither receptor is active; however, co-expression of receptors upon both ligands binding provides the dimerization of these receptors and therefore, these mutant receptors became functional (Maggio, Vogel, & Wess, 1993).

1.3.1.1 Fluorescence (Förster) Resonance Energy Transfer

Fluorescence (Förster) Resonance Energy transfer, firstly formulated by Theodor Förster in 1948, is a physical process depending on non-radiative energy transfer from donor molecular chromophore excited by an external light source to acceptor chromophore (Förster, 1948). The requirements of non-radiative energy transfer, that is no photon emitted or absorbed by donor and acceptor, respectively, are: the overlap of emission spectrum of donor chromophore between absorption spectrum of acceptor chromophore and also suitable orientation between donor and acceptor molecules, that is the distance between them is from 10 Å to 100 Å (Clegg, 1995). The distance between chromophores and orientation of them determine the efficiency of energy transfer and therefore, FRET efficiency (% E) changes with the 6^{th} power of the distance between the distinct fluorophores according to Förster formula (Förster, 1948):

$$\%E = Ro^6 \times 1 / (Ro^6 + r^6)$$

Where Ro (Förster distance where energy transfer is 50%) being specific value given for set of acceptor and donor chromophores; r being the distance between fluorophores.

Generally, three different approaches can be used to measure the FRET efficiency. Two of them depend on quenching of the donor and the third is related to enhanced fluorescence of the acceptor molecule. Quenching of donor will be performed in two ways. First one is a reduction in fluorescence density of the donor and second one is a decrease in lifetime of the donor in excited state. Another approach includes a raise in fluorescence intensity of the acceptor molecule by enhancing fluorescent protein (Stryer *et al.*, 1978).

Unlike other methods, FRET provides some advantages in live cell imaging. Firstly, the protein-protein interactions in intact cells can be detected by means of FRET. Secondly, subcellular localization of interaction can be visualized (Herrick-davis, Grinde, & Mazurkiewicz, 2004). Moreover, FRET is more suitable to observe membrane protein trafficking and interactions.

Comparing other methods, FRET is an effective method to observe a variety of interactions in the cell membrane. Moreover, FRET can be favored to visualize interactions of membrane proteins with themselves or other proteins. Besides, topological information of multimeric membrane proteins whose subunits are tagged by fluorescent proteins can be determined by FRET (Clegg, 1995).

There are alternative FRET techniques such as time-resolved FRET (TR-FRET) and photobleaching FRET (pbFRET). Firstly, pbFRET depends on the comparison between donor fluorescence intensities in

the cell before and after disrupting the acceptor fluorescence molecule via photobleaching. FRET is detected according to an increase of donor fluorescence on photobleached acceptor. The main problems in pbFRET are the background fluorescence, which arises from cellular autofluorescence, and a decrease in the signal to noise ratio. However, use of TR-FRET can be a solution for these concerns since TR-FRET involves the measurement of effect of an acceptor molecule on decay profile of a donor molecule that is excited with a short laser pulse (Klostermeier & Millar, 2002). As a result of short laser pulse, cellular autofluorescence will be decreased when compared with that after long-term laser exposures and also there is an increase in the signal to noise ratio. The advantage of this derivative FRET is being independent of concentration of donor and acceptor molecules; on the other hand, continuous excitation damages the fluorophore so that same sample cannot be analyzed for a long time (K. M. Kroeger *et al.*, 2001).

The most commonly used fluorescent protein in literature is green fluorescent protein (GFP) from Victoria aequrea, jellyfish discovered by Shimomura and his colleagues (Shimomura, Johnson, & Saiga, 1962). GFP and its colored variants, which have a wide range of spectral properties, are used as FRET pairs in many studies. At structural level, a choromophore within a molecule, which is responsible for color of a molecule of GFP, is p-hydroxybenzylideneimidazolinone in native protein (Cody, Prasher, Westler, Prendergast, & Ward, 1993) (Fig. 1.5).

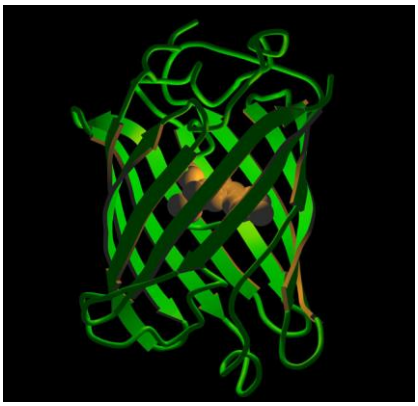

Figure 1.5 Structure of Green Fluorescent Protein (GFP)

In many studies involving GPCR dimerization, the variants of GFP, mainly cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), have been used as donor and acceptor chromophores, respectively. (K. M. Kroeger *et al*., 2001). For example, the dimerization of yeast α-factor receptor (Overton & Blumer, 2000), dopamine D2 receptor (Wurch, Matsumoto, & Pauwels, 2001) complement C5A receptors (Floyd *et al*., 2003) and neuropeptide Y receptor dimers (Dinger, Bader, Kóbor, Kretzschmar, & Beck-Sickinger, 2003) have been detected via FRET method using CFP and YFP. Besides CFP and YFP, red fluorescent protein (RFP) and green fluorescent protein (GFP) have been used extensively in FRET studies (Latif, Graves, & Davies, 2002).

Recently, other FRET pairs such as enhanced GFP (EGFP), and mCherry, a derivative of a red fluorescent protein find use in many studies (Shaner *et al.*, 2008). EGFP has large Strokes shift and good brightness

whereas mCherry has high photostability and large absorption cross-section; therefore these properties make them a good FRET pair (Albertazzi, Arosio, Marchetti, Ricci, & Beltram, 2009).

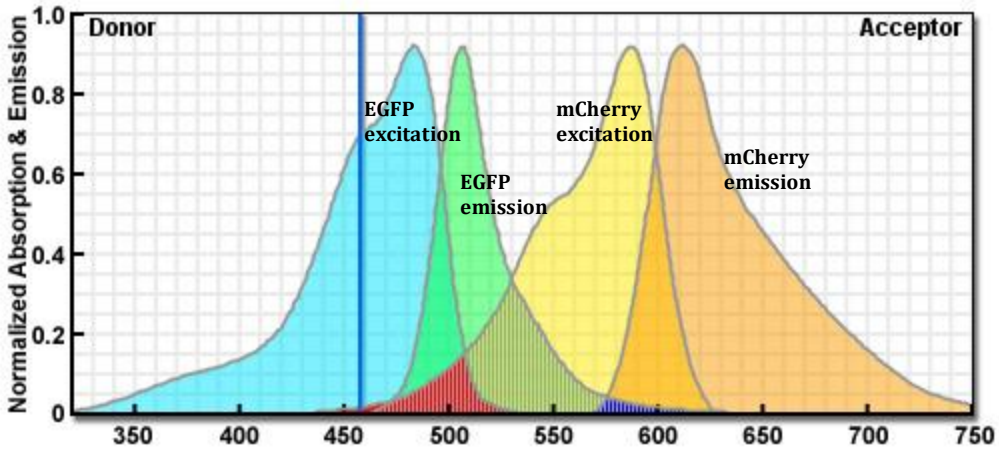

Figure 1.6 Spectroscopic characteristics of EGFP (cyan-green) and mCherry (yellow-orange) (drawn with FRET for Fluorescent Proteins JAVA Tutorial provided by MicroscopyU of Nikon®)

One of the requirements of being good FRET pair is narrow emission overlap region (Fig.1.6). Spectroscopic properties of EGFP and mCherry show that blue shaded part indicate the overlap of emission of mCherry and EGFP molecules which is narrow enough for minimal bleed-through. The laser we choose to excite EGFP is at 458 nm (shown as a blue line on Figure 1.6), which excite mCherry at less than 1% according to absorption spectrum of mCherry, thus minimize the direct excitation of acceptor molecule. Lastly, the overlap between absorption spectrum of donor and excitation spectrum of acceptor molecule between EGFP and mCherry is suitable for high FRET efficiency. Considering these properties, EGFP and mCherry forms a good FRET pair.

1.4 Methods to Screen Functionality of G-Protein Coupled Receptors

G-Protein Coupled Receptors (GPCRs) have an important role in biological pathways thus they are targeted by many drugs in the market. GPCRs are capable to recognize several endogenous stimuli such as ions, lipids, proteins and peptides (Fredriksson *et al.*, 2003). Predominantly, GPCRs utilize the mechanism to transduce extracellular stimuli to intracellular environment via heteromeric G proteins, which are coupled, to GPCRs (Bourne, 1997; Cabrera-Vera, 2003). In different subcellular localization and cell types, as a result of coupling to G protein, GPCRs generate distinct cellular responses (Thomsen, Frazer, & Unett, 2005). The screening techniques for GPCRs, especially second messenger assays, have been essential to facilitate drug screening (Thomsen *et al.*, 2005).

The assay involving the measurement of cAMP level is a common method to screen functionality of GPCRs. As secondary messenger, cAMP is included in the transduction of signal from extracellular environment. As a result of coupling of GPCR with Gαi/o and Gαs, the production of cAMP is either inhibited or stimulated, respectively. AC stimulates cAMP production from ATP molecule, thereby increasing cAMP level which leads the PKA activation thus phosphorylation of many substrate proteins in the cell (Babcock, Farzan, & Sodroski, 2003) assays have been designed to measure the cAMP level in the cell generated by GPCRs. Depending on regulation of AC by Gαi/o and Gαs subunits, there are several ways to determine cAMP level in the cell. Some of them include the competition between cellular cAMP and radioactively labelled cAMP for binding site of cAMP antibody. These assays use an antibody to a fluorescein-labeled cAMP tracer and cAMP. Competition between cellular cAMP and a tracer cause a reduction of polarization value with an increase in cAMP level (Von Euler, Sexton, Christopoulos, & Hick, n.d.).

Similarly, IP accumulation assays have been used to screen for Gq-coupled GPCRs. The activation of Gq of G protein stimulates PLC activation, thereby leading the hydrolysis of phosphatidyl-inositol-4, 5-bisphosphate (PIP2) that is a phospholipid in the plasma membrane. As a result of hydrolysis of PIP2, IP3 and diacylglycerol (DAG) are produced. Whereas IP3 affect intracellular Ca^{+2} mobilization, DAG induces PKC activation. So, the IP3 accumulation has been measured for GPCR function. Generally, biotinylated IP3 have been used to compete with cellular IP3 produced in the cell upon ligand binding *(*Von Euler *et al*., n.d.).

Another commonly used assay is intracellular calcium assays. Beside GPCR activated regulation, muscle contraction, and neurotransmitter release from synapses and apoptosis change calcium ion concentration in the cell. The binding of IP3 to IP3 receptors located on the ER membrane stimulates the release of Ca^{+2} from endoplasmic reticulum (ER). It is known that IP3 is produced as a result of hydolysis of PIP2 into two molecules IP3 and DAG, upon coupling of Gq subunits (Kirk, Maccallum, Michell, & Barker, 1990). Calcium indicators including Fura-2 and visible-light -excitable indicators show the calcium concentration when binding the Ca^{+2} in the cell (Hougland, Kravchuk, Herschlag, & Piccirilli, 2005).

MAPK cascades associated assays can also be used as an alternative to screen GPCR function. The activation of MAPK kinase (MEK) and extracellular signal-regulated kinases 1&2 (ERK1/2) are mainly regulated by Gq-coupled receptors, as a result of an increase in the level of intracellular DAG and Ca^{+2} through PKC. Phosphorylation of ERK1/2 is regulated by βγ subunits of Gi/o -coupled receptors (Kolch *et al.*, 1993*)*. Moreover, G_s subunit has effect on the phosphorylation of ERK1/2 through cAMP/PKA/B-Raf-dependent mechanism (Schmitt & Stork, 2002a, 2002b). As a result of involvement in multiple pathways, very specific antibodies used to detect phosphorylated ERK1/2 by Western Blotting and also enzyme -linked immunosorbent assay (ELISA) – based assay (Von Euler *et al*., n.d.).

When carrying out the functional screen assays, there are some critical aspects. The most important one is the condition of the cells used in the assay. GPCR of interest should be expressed in the cell lines at a reasonable level. GPCR can be transfected by two ways including stable and transient transfection method. High expression level can be obtained from transient transfection so, it is preferred in functional assay protocols. Cell lines used for assays are also important for G–protein and GPCR coupling process. Moreover, nonphysciological pathways can affect the overexpression of GPCRs in the cells. Cell density and controls, positive and negative, are important for all assays. Cell density can change strength of signal and background noise level whereas controls determine basal level of the measurement. Finally, non-specific binding of agonist to overexpressed GPCR can cause signal amplification, thereby leading a decrease in signal to noise ratio (Von Euler *et al*., n.d.).

1.4.1 Cyclic Adenosine Monophosphate (cAMP) -Glo Assay

The cAMP-Glo[tm] assay, commercially available by Promega Corporation, is based on the change in cAMP level in the cells. This assay can be used in order to monitor cAMP production upon agonist or test compound binding to GPCRs (Fig.1.7). When GPCRs are coupled to stimulatory G protein, $G_{\alpha s}$, activation of AC results in an increase level of cAMP, on the other hand GPCRs which are coupled to inhibitory, $G_{\alpha i}$, induce a decrease cAMP level by inhibiting AC activation. Mainly, the principle of this assay depends on the change in cAMP level affecting PKA holoenzyme activity, thereby leading a change of available ATP level in the cell and an increase or decrease in light production in luciferase reaction.

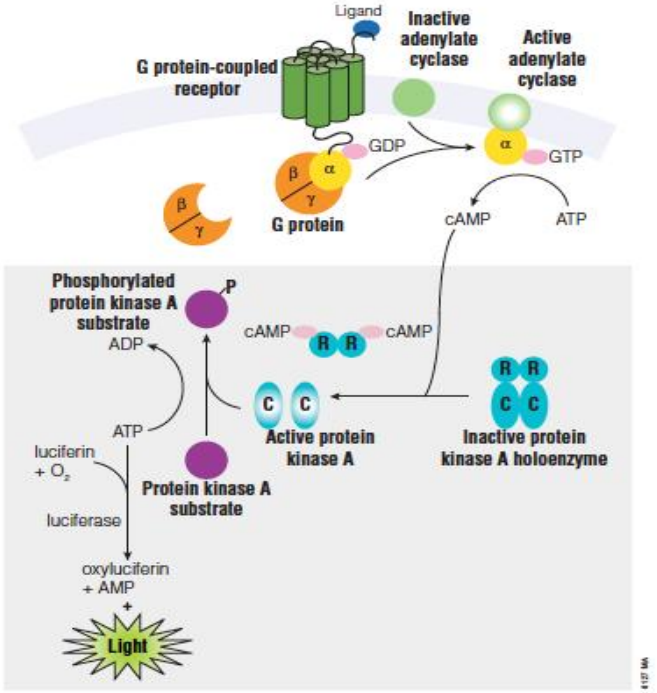

Figure 1.7 Schematic diagrams of cAMP production in cells upon ligand binding and the cAMP-Glo™ Assay (taken from www.promega.com)

Protein kinase A includes four subunits, two catalytic and two regulatory subunits. Binding of cAMP molecules to regulatory subunits of PKA converts inactive state of PKA to active state, as a result of conformational change of holoenzyme. Active state of PKA including catalytic subunits regulates the reaction, which is transfer of last phosphate group of ATP to substrate of PKA. As a result of consumption of the ATP, ATP level involved in the light production will decrease. Thus, luminescence intensity and cAMP level in the cells have reciprocal relationship in this assay.

1.5 Aim of the Study

Adenosine A2A and dopamine D2 receptors both affect neurophysiological events and are targeted by neuropsychiatric drugs. Thus, their physical interaction and mechanism of this interaction may reveal new approaches to explain both physiology and pharmacology of these receptors in more detail as well as to pharmacologically target them more efficiently.

The purpose of this study is to assess the functionality of EGFP (enhanced green fluorescent protein) and mCherry (a red fluorescent protein) tagged adenosine A2A and dopamine D2 receptors and to qualitatively assess the dimerization of these receptors in live cells via Fluorescence Resonance Energy Transfer (FRET) method. Understanding the mechanisms of the interaction between adenosine and dopamine signaling will help us to figure out some molecular mechanism of neurophysiological disorders. Furthermore, the fluorescence based live cell model could be used to observe the effects of potential drugs on the interaction of these two receptors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Neuro2a (N2a) Mouse Neuroblastoma Cell Line and Media

Neuro2a (N2a) mouse cell line was transiently transfected in order to express and visualize the receptors tagged with fluorescent proteins. This cell line was provided by ATCC and kindly granted by Assist. Prof. Dr. Tülin Yanık from METU Biological Sciences, Ankara, Turkey.

Growth medium used to sustain N2a cells was Dulbecco's Modified Eagle Medium (D-MEM) (50%) supplemented with L-glutamine (Invitrogen, Cat#41966029) and OptiMEM®I Reduced Serum Medium with L-glutamine (Invitrogen, Cat#31985047) (50%) , Fetal Bovine Serum (Invitrogen, Cat#26140-079) (10%) and Penicillin/Streptomycin solution (Invitrogen, Cat#15140-122) (1%). Millipore Stericup® Filter Units was used for sterilization step to filter the final solution.

10X Phosphate Buffered Saline (PBS) was prepared and was diluted to 1X with distilled H_2O in order to wash cells to get rid of the dead cells and rest of the waste medium. The content of PBS was given in Appendix D.

TrypLE™ Express Stable Trypsin-Like Enzyme with Phenol Red (Invitrogen, Cat#12605-028) was used for detachment of cells from culture flasks surface during their passage.

Freezing medium was prepared to freeze the cells to make stock cells. This medium was composed of 35% D-MEM, 35% OptiMEM®I, 20% glycerol (as cryoprotectant) and 10% FBS. Around 10^7 N2a cells were frozen following a confluency of 80-90 % was observed.

Cell culture equipment including all reagents and chemicals were cell culture grade.

2.1.2 Neuro2a Cell Culture Conditions

The cells were grown at 37°C in a 5% CO2 atmosphere provided by Heraeus® Hera Cell 150 Tri- Gas Cell Culture incubator. Cell culture experiments were performed under sterile conditions using a laminar flow cabinet with a hepa filter.

2.1.3 Bacterial Culture Media and Conditions

Luria Bertani (LB) medium was used to reproduce the Escherichia coli TOP10. The content of LB was indicated in Appendix C. Medium compositions were mixed in distilled water (dH_20) and pH of mixture was adjusted to 7.0 by addition of 1 N KOH. After preparing the medium mixture, it was autoclaved at 121°C for 20 minutes for sterilization. LB agar containing extra 1.5 % agar was supplemented with either 100 mg/ml ampicillin or 50 mg/ml kanamycin prepared for bacterial selection part of experiments. Cooled medium was mixed with antibiotic solution. Solid LB agar plates were incubated at 37°C in a Nüve® brand incubator. Selected colonies were grown in liquid LB at 37°C in a Zheiheng shaker incubator.

Bacterial stocks were prepared by mixing liquid culture and 50 % glycerol ata ratio of 50:50 to make final concentration of glycerol 25%. Stocks were kept at -80°C in the freezer.

2.1.4 Other Chemicals and Materials

The chemicals were supplemented from Sigma Chemical Company (NY, USA), Applichem (Darmstadt, Germany) and Sigma Aldrich (Taufkirchen / Germany). Molecular biology kits were from Fermentas (Ontario, Canada), QIAGEN (Düsseldorf, Germany) or Invitrogen (CA, USA). DNA polymerases used in polymerase chain reactions (PCR) were from Fermentas (Ontario, Canada), from New England Biolabs (Hertfordshire, UK) and from Finnzymes (Vantaa, Finland). Cell culture media and reagents were all from GIBCO®, Invitrogen (CA, USA). Functional assay kits were purchased from Promega (WI, USA). Agonist of Dopamine D2 receptor, namely Quinpirole, was a kind gift by Assist. Prof. Dr. Hakan Kayır from GATA, Department of Medical Pharmaceuticals, Ankara.

T-75 cell culture flasks and 35 mm cell culture dishes from Greiner (Frankfurt, Germany) were used for cell culture experiments and functional assay protocol. Sterile serological pipettes were purchased from LP Italiana (Milano, Italy). Glass bottom dishes ordered from In Vitro Scientific (CA, USA) were used for transfection and live cell imaging experiments. For functional assay, 96 well plates were purchased from BD (Becton, Dickinson & Company, NJ, USA).

Live cell-imaging experiments were performed using a Zeiss LSM 510 confocal laser scanning microscope (Fig 2.1). Expression control experiments were performed using a Leica fluorescence microscope.

Figure 2.1 Zeiss 510 LSM model (taken from http://unam.bilkent.edu.tr/confocal_microscope.html)

2.2 Methods

2.2.1 Preparation of Competent E.coli Cells by RbCl2 methods

Top 10 E.coli stock was streaked onto LB agar plate without antibiotic selection and was incubated overnight at 37 °C. After 24-hour incubation, a single colony picked from the plate, was inoculated into 5 ml culture of liquid LB. This culture was incubated by shaking overnight at 37 °C. Next day, the overnight culture was added to 100 ml culture of LB in 250 ml Erlenmeyer flask and was grown by shaking for about 2 hours until OD reach to 0.5. Bacterial culture was then divided into 50 ml falcon tubes that were chilled on ice for 5 minutes followed by centrifugation at 6000 rpm for 5 minutes at 4 °C. The supernatant was discarded and the cells were resuspended in 2/5 volume of transformation buffer I. The pellet was dissolved gently and chilled on ice for 5 minutes and then centrifuged at 6000 rpm for 5 minutes at 4 °C. The supernatant was decanted and cells were resuspended in 1/25 original volume of transformation buffer II. The ingredients of transformation buffers were given in Appendix D. The dissolved cells were chilled on ice for 15 minutes. Finally, 100 µl aliquots of the suspension was added into 1.5 ml eppendorf tubes, and stored at –80 °C.

2.2.2 Transformation of Competent E.coli cells with a plasmid

The competent E.coli cells prepared by $RbCl_2$ were used for transformation of plasmids. Firstly, cells taken from –80 °C freezer were chilled on ice for 10 minutes in order to defreeze cells. After 10 minutes, desired amount of solution containing plasmid was added into eppendorf tubes containing competent cells. This amount changes between 50 ng and 100 ng. Competent cells containing plasmids were chilled on ice for 30 minutes. The critical step of the transformation is heat shock. This step provides the transformation

of plasmids into competent cells by widening the pore of cell membrane of cells, thereby making it easier for the plasmids to be taken in. The competent cells incubated for 30 minutes were placed on heat block, and the temperature was adjusted to 42 °C, for 90 seconds. Immediately, cells were taken and incubated on ice for 5 minutes. In order to grow the cells, preheated 900 ul sterile LB was put on cells. 1.5 ml eppendorf tubes containing competent cells and LB were incubated at 37 °C in shaker incubator. After cells were incubated for 1 hour, they were spun at 6000 rpm for 3 minutes in the centrifuge. 800 ul supernatant was discarded without destroying the pellet containing cells. By resuspending pellet containing with remained 200 µl supernatant, the pellet was inoculated onto LB agar plate with selected antibiotic. In order to spread the cell mixture, sterile glass beads were used. Inoculated LB agar plates were incubated overnight at 37 °C in incubator. The whole procedure was carried out under sterile conditions.

2.2.3 Plasmid Isolation from E.coli cells

Transformed E.coli cells, which were grown on the selected LB agar plate, were inoculated to 5 ml liquid LB broth mixed with 5 µl of selected antibiotic solution by picking single cells. They were incubated overnight at 37 °C in shaker incubator. After overnight incubation, plasmids were isolated from 5 ml of bacterial inoculum by using Fermentas® GeneJET™ Plasmid Miniprep Kit and following the protocol mentioned in instructor`s manual.

2.2.4 Polymerase Chain Reaction (PCR)

Previously prepared constructs containing fluorescent protein tagged receptors were transformed and isolated to obtain reasonable amount of plasmids for transfection part. To check the fluorescent proteins genes, which have already been fused to receptors optimized PCR protocol was applied (Table 2.1). The primers are given in Appendix G.

Table 2.1 Optimized PCR conditions for amplifying fluorescent protein genes

Ingredients	Volume
Nuclease free water	33 µl
Phire® Reaction Buffer (5X)	10 µl
$MgCl_2$ (25 mM)	1 µl
dNTPs (25mM)	1 µl
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template	1 µl
Phire® Hot Start II DNA Polymerase	1 µl
DMSO	1 µl
Total	50 µl

Cycle parameters

98°C for 30 sec
35 cycles of;
    98°C for 30 sec
    55°C for 5 sec
    72°C for 15 sec
72°C for 1 min
4 °C forever

2.2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to control the size of fluorescent protein genes, amplified by PCR method. 1 % w/v agarose gel was used to run the PCR products. Gel was prepared by using 1X TBE buffer, given in Appendix D. After cooling the gel down, required volume of 10 mg/ml EtBr stock solution was added into gel solution to staining DNA. EtBr is used as a DNA interchelator. To check size of genes, appropriate quick load DNA size ladders were loaded and at loading step, PCR products were mixed with 6X DNA loading dye (Fermentas®, Cat#R0611, given in Appendix D) to obtain DNA: dye ratio as 5:1 and then loaded to gel. The horizontal electrophoresis tank containing 1X TBE was used to run the gel at 120 V for 20-25 minutes. The gel was visualized on UV transilluminator modified with camera systems.

2.2.6 Extraction of DNA from Agarose Gel

At the end of the digestion and PCR, the products were run on agarose gel. These products were extracted by using QIAGEN® Gel Extraction Kit (Cat# 28704). The excised bands were eluted by following the protocol mentioned in instructor`s manual.

2.2.7 Quantification of DNA Concentration

After plasmid isolation and gel extraction protocol, the concentration of DNA was quantified by using NanoDrop 2000 spectrophotometer from Thermo Scientific® brand. After loading of 1µl of solution containing eluted DNA onto the micro-volume pedestal, the measurement was taken according to user's manual instructions.

2.2.8 Maintenance of N2a Neuroblastoma cell lines

N2a cells were sub-cultured after reaching to 80-90 % confluence taking approximately 3 days. At every three-day, medium in the flask was aspirated and cells were washed with 1X PBS once. At the end of

washing step, 1 ml of trypsin solution were added and incubated for 5 minutes at 37 °C to detach the cells. After 5 – minute incubation, detached cells were diluted with 9 ml of growth medium. Cells were diluted 10 fold. 1 ml of cell suspension was transferred into T-75 culture flask containing 14 ml growth medium.

The cells reaching to 90 % confluence were counted and about 10^7 cells were centrifuged at 1000 rpm for 5 minutes. After centrifugation, the cells, which precipitated, were resuspended with 1 ml freezing medium and transferred into cryovial tubes. These tubes were kept in –80 °C freezer for 24 hours and then were stored at about –150 °C in a liquid nitrogen tank. To culture from the frozen stock cells, frozen cells were thaw at room temperature and transferred in 4 ml growing medium. This mixture was centrifuged at 1200 g for 10 minutes. The precipitation containing cells were resuspended with 5 ml growing medium and transferred to cell culture flask containing growth medium.

2.2.9 Cell counting

At the end of sub-culture, cells were counted for freezing and seeding on dishes. 10 µl of cell suspension applied onto a heamocytometer. Cells in 4 corners (composed of 16 small squares) with 1 mm^2 area were counted under a light microscope and the cell number was calculated by the formula:

(Total cell number in 4 big squares x 10^4) /4 = number of cells per ml

Working cell concentration was adjusted to 100.000 cells/ dish for FRET sets and functional assay.

2.2.10 Transfection of N2a neuroblastoma cells with pcDNA 3.1 (-) eukaryotic expression vector

Lipofectamine™ LTX with Plus™ reagents from Invitrogen® was utilized to transfect plasmid to eukaryotic cells and to increase the transfection efficiency. The day before transfection experiment, N2a cells were counted. The cells were counted with haemocytometer and a volume of medium containing 100.000 N2a cells was dropped on 35 mm culture dish, where coverslips were inserted, and on a glass bottom dish. To adjust the final volume to 2 ml, needed volume of growing medium was added to dish. The cells were incubated at 37 °C, 5 % CO_2 for 24 hours. After 24-hour incubation, transfection protocol was carried out. Firstly, the mixture containing 200 ng of plasmid to be transfected, 100 ml of OptiMEM® I and 6 µl of Plus™ reagent was prepared in 1.5 ml eppendorf tube. In the case of double transfection, 200 ng of each plasmid was diluted in 100 ml of OptiMEM® I. This mixture was incubated for 15 minutes at room temperature. During the 15-minute incubation, in another tube, 100 ml OptiMEM® and 4 µl of Lipofectamine LTX™ were mixed. At the end of 15-minute incubation, this mixture was added to previously prepared mixture containing plasmid DNA, Plus™ in OptiMEM® I. This final mixture was incubated for 15 minutes at room temperature. In the meantime, the medium in a dish was removed and the cells were washed with 1 ml of sterile 1X PBS. After discarded PBS, 1 ml of OptiMEM® I was put on the cells in the dish. At the end of last 15-minute incubation of transfection mixture, the mixture was dripped on the cells. After complete the transfection protocol, the incubation step was applied during next 3 hours in the 5% CO2 incubator at 37 °C. After 3-hour incubation, in order to grow transfected cells, 2.5 ml of normal growth medium containing serum was put on the dish. The cells were incubated for 48 hours for the expression of the constructs in N2a cells in the 5% CO2 incubator at 37 °C.

2.2.11 cAMP Functionality Test

To check the functionality of fluorescent-tagged receptors, Promega cAMP-Glo™ Assay was used. CGS21680 (2-[p-(2 carboxyethyl) phenethylamino]-5'-N-ethylcarboxamidoadenosine), and Quinpirole from Sigma Aldrich were used as agonist of adenosine A2A and dopamine D2 receptors, respectively. Moreover, forskolin from Sigma Aldrich, which increases cAMP level in the cell, was used for dopamine receptor experiments. Before carried out the assay protocol given in the user`s manual, 30.000 N2a cells were seeded on the 35 mm culture dish. These transfected and untransfected cells were incubated for 24 hours in the 5% CO_2 incubator at 37 °C. Next day, the medium on the cells was discarded and cells were rinsed with 1X PBS. Removed PBS, 0.5 ml of trypsin was applied to detach the cells for 5 minutes at 37 °C. After that, 2.5 ml of growth medium was added to inactive the trypsin. Cells containing medium was counted and 5000 N2a cells were transferred to poly D-lysine coated 96 well - plate. Remaining cells in the medium were kept on coverslip inserted in 35 mm culture dish to check the expression of tagged receptors by imaging method. In order to attach the cells, 96 well plate and dishes were incubated overnight in the 5% CO_2 incubator at 37 °C. After overnight incubation, functional assay protocol was applied. According to assay protocol, to generate the standard cAMP curve in triplicate, 4 µM cAMP solution was prepared by dilution of 1 mM cAMP stock solution with 1X induction buffer. The content of induction buffer is given in Appendix D. The agonists were also dissolved in 1X induction buffer. Another 96 well- plate was used to make 2X serial dilution of cAMP solution with induction buffer, and then 20 µl of cAMP solution was transferred to 96 well -plate. Meanwhile, the medium was discarded from the wells and 20 µl of agonist solution including 10 µM quinpirole and 1.5 µM CGS21680 and induction buffer was added to treated group and untreated group, respectively. In the case of functional of D2R, there was additional step before agonist treatment. 20 µl of 10 µM forskolin was added to all groups and the cells were incubated for 15 minutes at 37 °C. After that, agonist of dopamine receptor was added to treated group. To let the agonist interact with the receptors, the cells were incubated for 15 minutes at 37 °C. At the end of 15–minute incubation period, the assay protocol was followed according to instructor`s manual. The measurement was done with luminometer.

2.2.12 Imaging Transfected N2a Cells with Laser Scanning Confocal Microscope

FRET analysis needs three parameters; therefore three transfection reactions were prepared. The cells were transfected with only EGFP tagged receptor as a donor, mCherry tagged receptor as acceptor and both EGPF and mCherry tagged receptors as a FRET. Donor sample and acceptor sample, which were transfected by single construct, were used to calculate spectral bleed –through (SBT) signal. This signal arises from the absorption of donor molecule in the acceptor channel and the excitation of acceptor molecules at excitation wavelength of donor molecules.

During FRET analysis, three different set-ups were used to calculate bleed through signals and FRET efficiency. Some parameters such as excitation and emission wavelength were same. The laser exciting at 458 nm was used to excite EGFP molecule and filter collecting signal over 585 nm was used to signal from mCherry molecule for FRET images. FRET images were taken from the same region of interest to normalize SBT. One of them was taken at EGFP channel, by exciting at 458 nm and collecting at the 505-580 nm region. For another image, the cells were excited at 543 nm, which can excite mCherry molecule, and the emission was collected over 585 nm. This set up was involved in mCherry signal. To calculate donor bleed –through, the images containing only EGFP tagged receptors were taken by exciting at 458 nm and collected at both channels (mCherry and EGFP). Differ from donor bleed –through, acceptor bleed –through was calculated by using the cells transfected only with mCherry tagged receptor, which were

excited at 458 nm and 543 nm. These emissions were collected over 585 nm, which is mCherry channel. These set-ups, also known as tracks, are given in Figures 2.2 and 2.3.

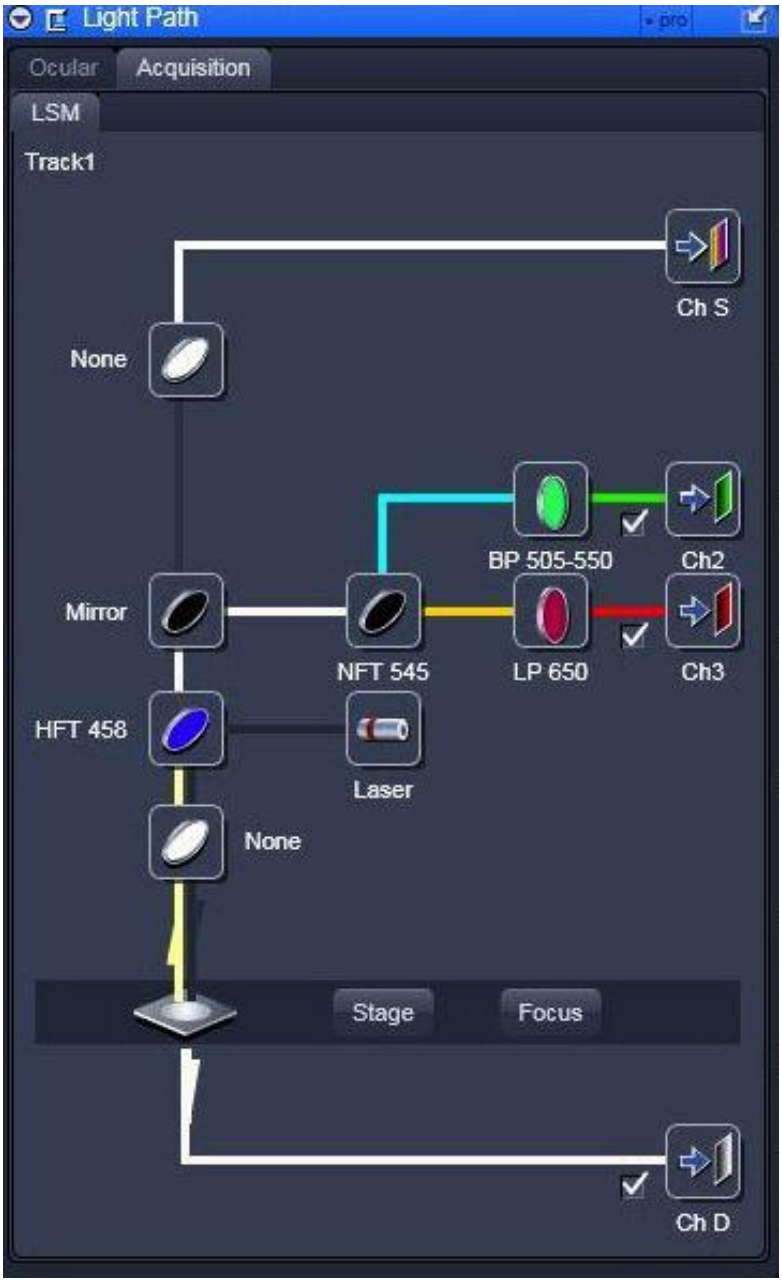

Figure 2.2 Track 1 configuration in laser scanning confocal microscope to detect FRET in channel 3 and EGFP signal in channel 2, after exciting the cell at 458 nm

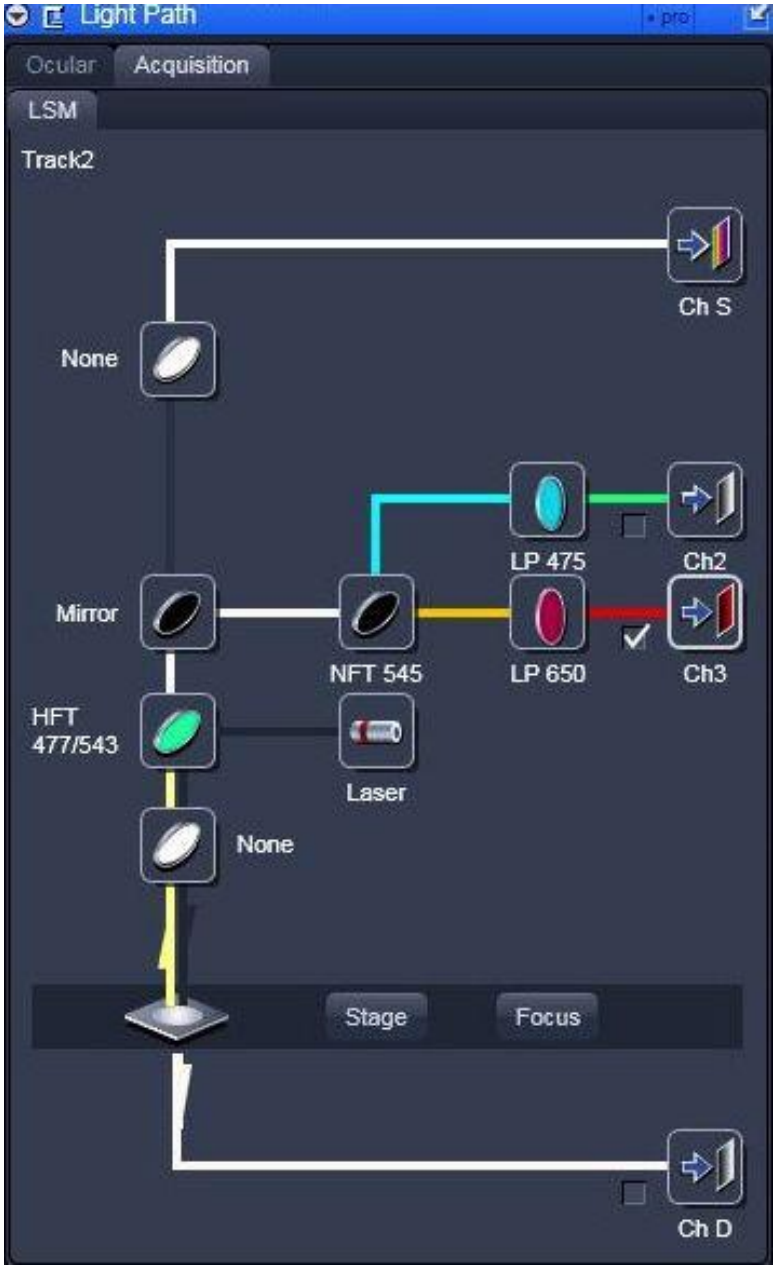

Figure 2.3 Track 2 configuration in laser scanning confocal microscope to detect mCherry signal in channel 2 after exciting the cell at 543 nm

FRET was calculated by using PixFRET plug-in of ImageJ software (Feige, Sage, Wahli, Desvergne, & Gelman, 2005).

2.2.13 Image Analysis with Pix-FRET

The normalization of spectral bleed –through (SBT) is the main problem in FRET analysis. As mentioned, SBT signal, that is a false positive FRET signal, can result from either the absorption of donor molecule in the acceptor channel or the excitation of acceptor molecules at excitation wavelength of donor molecules. In order to handle this problem, Pix-FRET plug-in in Image J that allows creating the images of sensitized –emission FRET can be used; therefore, it can be possible to determine wherever FRET occurs by counting up pixel-by-pixel the images visualized in three channel settings.

Firstly, images were taken from the cells transfected with only donor, EGFP tagged receptor, to calculate donor bleed-through by following setups:

- excitation of the donor and emission of the acceptor
- excitation of the donor and emission of the donor

Secondly, for acceptor bleed-through, only acceptor, mCherry tagged receptor, was transfected to cells and images taken with the following setups were used;

- excitation of the donor and emission of the acceptor
- excitation of the acceptor and emission of the acceptor

Lastly, two fluorophores must be present at the same time and three images were taken with the following setups;

- excitation of the donor and emission of the acceptor
- excitation of the donor and emission of the donor
- excitation of the acceptor and emission of the acceptor

Using bleed-through images, Pix-FRET outputs three line equations; constant, linear and exponential; that fit and comprise most of the pixel intensity values.

The best equation from these three was chosen to normalize the bleed-through. Afterwards, FRET image is analyzed according to this normalization.

2.2.14 Statistical Analysis

Student`s t-test was conducted between control (or untreated) versus treatment groups (two-tailed unpaired comparison). p values that were <0.05 were considered as statistically significant throughout these studies.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Confirmation of Fluorescent Protein Genes

The wild type and fluorescence protein tagged forms of Adenosine A2A and Dopamine D2 receptor were cloned into mammalian cell expression vector, pcDNA 3.1 (-) in order to be used in transfection reaction into N2a cells. The sequences of fluorescent protein tagged receptors and maps of backbone plasmid are given in Appendix E. The fluorescent protein genes including EGFP and mCherry genes were previously fused to C-termini of the wild type receptors by PCR integration method as detailed in the materials and methods part of the thesis "Optimization of FRET Method to Detect Dimerization of Dopamine D2 and Adenosine A2A Receptors in Live cells" by Gökhan Ünlü. Briefly, PCR integration method consists of two tandem PCR reactions where the first PCR reaction amplify the fluorescent protein genes with overhangs that was determined by the target locations. According to the locations, 5` of forward primer included the last 30 bp of the receptors without stop codon; on the other hand, 5` of reverse primer included 30 bp of the vector sequence directly following the sequence of the receptor. For PCR integration reaction, the PCR products amplified in first PCR reaction and pcDNA 3.1 (-) holding receptor genes were used as the primer set and the template, respectively in the second PCR reaction. At the end of the PCR integration reaction, a new full vector carrying fused receptor gene was amplified. These vectors were used in order to test the functionality of these receptors and test dimerization of them. Therefore, the newly isolated plasmids were obtained after transformation steps.

Isolated plasmids were checked whether fluorescent protein genes were inserted or not by again using PCR. Fluorescent protein genes were amplified by PCR to confirm the presence of EGFP and mCherry genes in isolated plasmids. Primers are given in Appendix E. At the end of reaction, PCR products were run on 0.8 % agarose gel as seen in Fig 3.1.1

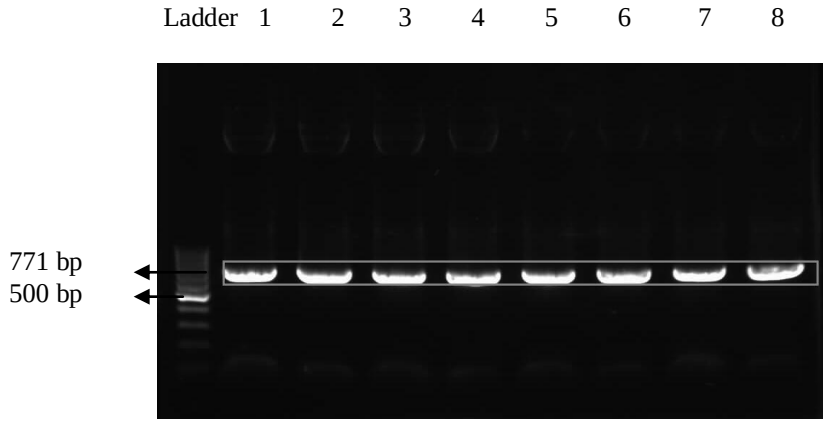

Figure 3.1.1 Agarose gel image of PCR product of mCherry and EGFP amplified from pcDNA 3.1(-) including fluorescent protein tagged receptors. Fermentas® GeneRuler™ 100 bp DNA Ladder was loaded to 1st well.

1. pcDNA 3.1 (-) _A2AR + mCherry#1
2. pcDNA 3.1 (-) _A2AR + mCherry#2
3. pcDNA 3.1 (-) _A2AR + EGFP#1
4. pcDNA 3.1 (-) _A2AR + EGFP#2
5. pcDNA 3.1 (-) _D2R + mCherry#1
6. pcDNA 3.1 (-) _D2R + mCherry#2
7. pcDNA 3.1 (-) _D2R + EGFP#1
8. pcDNA 3.1 (-) _D2R + EGFP #2

Isolated plasmids including fluorescent protein tagged receptor genes were checked and complementing pairs of theses plasmids were transfected to N2a cells for FRET analysis and functional assays.

3.2 Functional Assessment of Fluorescent Protein Tagged Receptors

To assess the functionality of EGFP and mCherry tagged receptors, the functional assay depending on cAMP levels produced in transfected cells in response to agonist binding was performed. The functionality of these receptors was evaluated in comparison to wild type (WT) and non-treated or mock transfected N2a cells. Moreover, the expression of receptors was visualized with fluorescence microscopy.

The cells were seeded on cover glasses inserted in 35 mm culture dishes. The required cell amount (5000 cells) was used for functional assay and the rest of cells were used to check expression of fluorescent protein fused receptor genes in N2a cells. Prior to the functionality tests, the cells were visualized with fluorescence microscopy (Fig. 3.2.1).

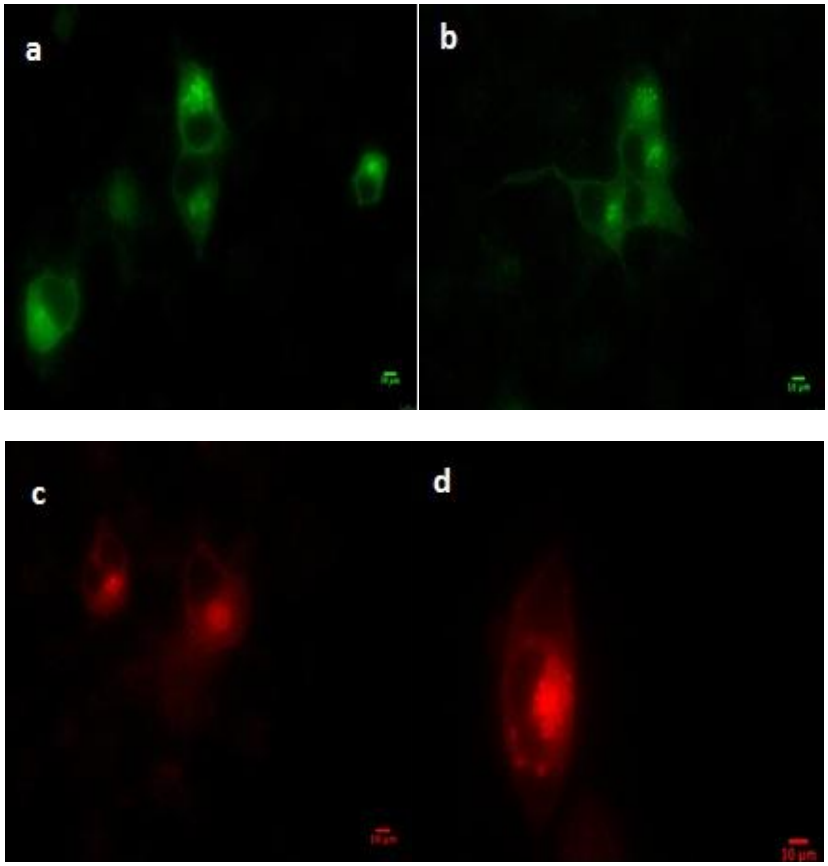

Figure 3.2.1 Fluorescence microscope images of N2a cells transfected with a) A2AR_EGFP and b) D2R_EGFP in pcDNA 3.1 (-) vector, excited at 458 nm and c) A2AR_mCherry and d) with D2R_mCherry in pcDNA 3.1 (-) vector, excited at 543 nm (100x oil objective)

When the fluorescent proteins fused receptors were transfected into N2a cells used for functionality test, the signals were detected from both the membrane and the cytosol of cells as seen in Fig. 3.2.1. Images taken by fluorescence microscopy used in this experiment differ from confocal microscopy images in that, signal is collected from all the layers of a cell and reflect these signals as a whole, therefore, fluorescence signal is not sharp and seems to be all over the cell body. Although this image is not sharp the aim is only to confirm the expression of transfected EGFP and mCherry tagged receptors- in N2a cells. Following a positive fluorescent signal, these constructs were used for both functional assays and dimerization studies that were imaged by confocal microscopy.

Firstly, the functionality of EGFP and mCherry tagged A2A receptors were assessed by cAMP production in response to CGS21680 agonist, which is a specific agonist for A2A receptor. N2a cells are mouse Neuroblastoma cells and contain wild type A2A receptors, therefore, this agonist in induction buffer was used to induce the activation of not only A2A receptors transfected into N2a cells, but also those in un-transfected and mock transfected N2a cells (which were used to normalize the results to see the functionality of only the transfected genes). N2a cells as treated group were subjected to agonist for a period of 15 min. prior to cell lysis. Finally, the change of cAMP level was detected in the cells.

cAMP is a secondary messenger involved in many pathways and cellular processes. It is hard to say that total change of cAMP level in transfected cells resulting from the agonist induced A2A activation. However, we assumed that the broad range phoshodiestrase, IBMX used in this study, present in induction buffer, which hydrolyzes cAMP in the cells before agonist treatment will eliminate this background signal. Moreover, N2a cells express the wild type A2A receptors. Addition of agonist leads the activation of these receptors upon ligand binding in transfected cells, as well as non-transfected cells. Therefore, cAMP level in basal demonstrates that possible cAMP production in cells and A2AR induced cAMP production is represented as percentage over basal levels (100%). It is observed that the wild type A2A receptors cloned to pcDNA 3.1 (-) vector were expressed into N2a cells and as a result of agonist treatment, in wild type A2A receptor transfected cells, the production of cAMP is three times more with respect to the production in non- transfected cells (Fig. 3.2.2). In the case of functionality of EGFP tagged A2A receptor, the production of cAMP is four times more with respect to the production in non- transfected cells. On the other hand mCherry tagged A2A receptors shows relatively less activity but was still significantly different from mock-transfected cells. Compared with non-transfected cells, there is a doubling of cAMP production in A2AR –mCherry transfected cells (Fig. 3.2.2). cAMP production in N2a cells transfected with both WT and tagged receptors were significantly increased compared to in untransfected cells (indicated as basla level). Moreover, there is no significant difference in cAMP production in WT and fluorescent protein tagged receptors. Tagged receptors seem to be as functional as wild type receptors. As a result, there was a significant increase in cAMP production upon CGS21680 treatment in transfected cells with WT- A2Areceptors and the A_{2A} receptors tagged with EGFP and mCherry proteins according to positive coupling of A2AR to the adenylyl cyclase.

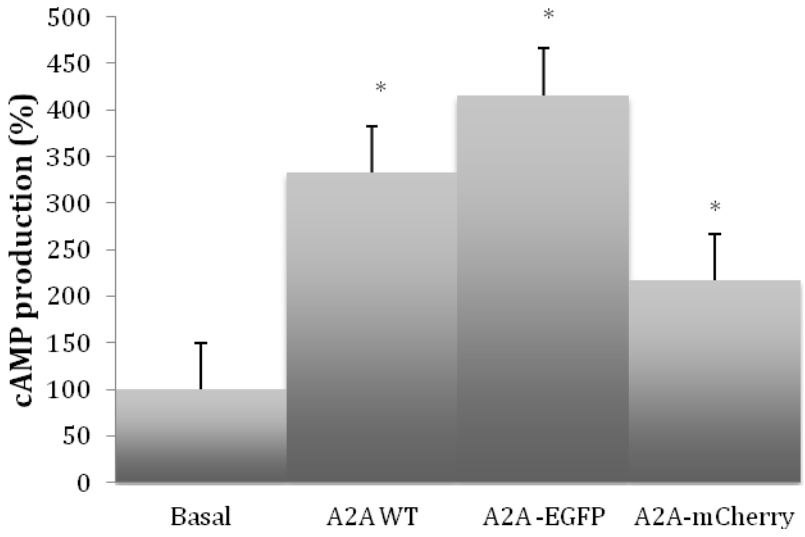

Figure 3.2.2 Functionality test of Adenosine A2Areceptor fused to EGFP and mCherry. N2a cells transiently transfected with 500 ng of wild type A2AR, A2AR-EGFP and A2AR-mCherry in pcDNA 3.1 (-) were stimulated with 1.5 µM CGS21680. A2AR induced cAMP production is indicated as percentage over basal levels (100%). Three independent experiments were performed in triplicate. Data is analyzed according to Student`s t-test and * indicates $p < 0.05$.

In contrast to the functionality test of A2A receptors, the test for D2 receptors require the pre-treatment step. In agreement with the inhibition of adenylyl cyclase by dopamine D2 receptor, cAMP production is directly proportional reduced in response to agonist binding to D2R. In order to measure the reduction in cAMP levels, cAMP production in cells were increased by forskolin treatment. By activating adenylyl cyclase and increasing cAMP levels, forskolin is able to resensitize cell receptors. For this purpose, the cells transfected with wild type D2R and fluorescent protein fused D2R were subjected to forskolin and incubated for 15 min (enough time to interact with cell receptors). To measure the effect of forskolin on the cells, non-transfected cells were also treated with forskolin. After forskolin incubation period, cells were treated with quinpirole to activate D2 receptors on membrane. In Figure 3.3.2, the activation of EGFP and mCherry fused D2 receptors diminished the cAMP production more than half of forskolin induced cAMP accumulation. Moreover, the overexpression of wild type D2R in transfected cells leaded the decrease of forskolin induced cAMP production. Therefore, in agreement with the inhibitory effect of D2 receptor on AC activity, quinpirole, the agonist of D2 receptors, was able to reduce cAMP levels induced by forskolin addition in N2a cells transfected with D2R-mCherry and D2R- EGFP (Fig. 3.2.3).

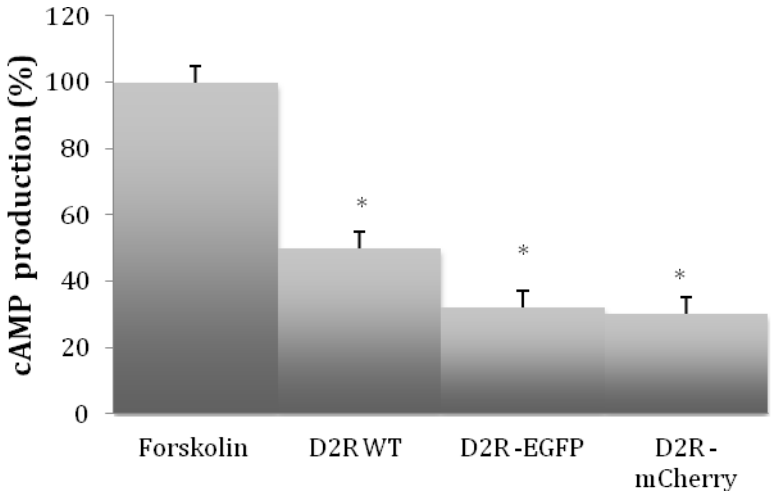

Figure 3.2.3 Functionality tests of Dopamine D2 receptors fused to EGFP and mCherry fluorescent proteins. N2a cells transiently transfected with 500 ng of wild type D2R, D2R-EGFP and D2R-mCherry in pcDNA 3.1 (-) were stimulated with 10 uM quinpirole in the presence of 10 µM forskolin. Results are represented as D2 receptor`s ability to inhibit forskolin induced cAMP production (100 %) in untransfected cells. Three independent experiments were performed in triplicate. Data is analyzed according to Student`s t-test and * indicates $p < 0.05$

3.3 Qualitative Assessment of Dimerization of Fluorescently Tagged Receptors via FRET

3.3.1 Homodimerization of Adenosine A2A receptors

In this study, A2A EGFP and mCherry tagged receptors were used to demonstrate the homodimerization of A2A receptors using FRET technique. A2AR homodimerization has not been comprehensively studied as much as heterodimerization. In some studies including FRET and /or BRET, C- terminally tagged A2A receptors have been used and the homodimerization of A2AR-A2AR have been detected (Canals, Burgueño, *et al.*, 2003). Similarly, the constructs used for A2A homodimerization were tagged at C-terminal tail of receptors in this study.

FRET sets were prepared for A2A homodimerization included three models, double transfected cells (EGFP and mCherry tagged receptors) as FRET model, only EGFP tagged receptor transfected cells as donor model and only mCherry tagged receptor transfected cells as acceptor model. For analysis, the images were taken at each channel including EGFP, mCherry and FRET channels. Firstly, to calculate the donor bleed-through signals from EGFP molecules, the cells transfected with only EGFP tagged A2A receptors- were used. The images of cells visualized at FRET channel and EGFP channel were stacked into one image. The images were given separately in Fig. 3.3.1. The transmission modes of cells were also given to indicate the morphologies of N2a cells. Similarly, Figure 3.3.2 gives the images used for acceptor bleed through calculation from cells transfected only A2A-mCherry plasmids. After defining these bleed through signals as baseline signals for FRET calculation, the samples including cells transfected with both constructs were prepared for FRET images. The channels of FRET images were stacked in the following order: FRET, donor and acceptor images. The representative images of these channels were given in Fig. 3.3.3

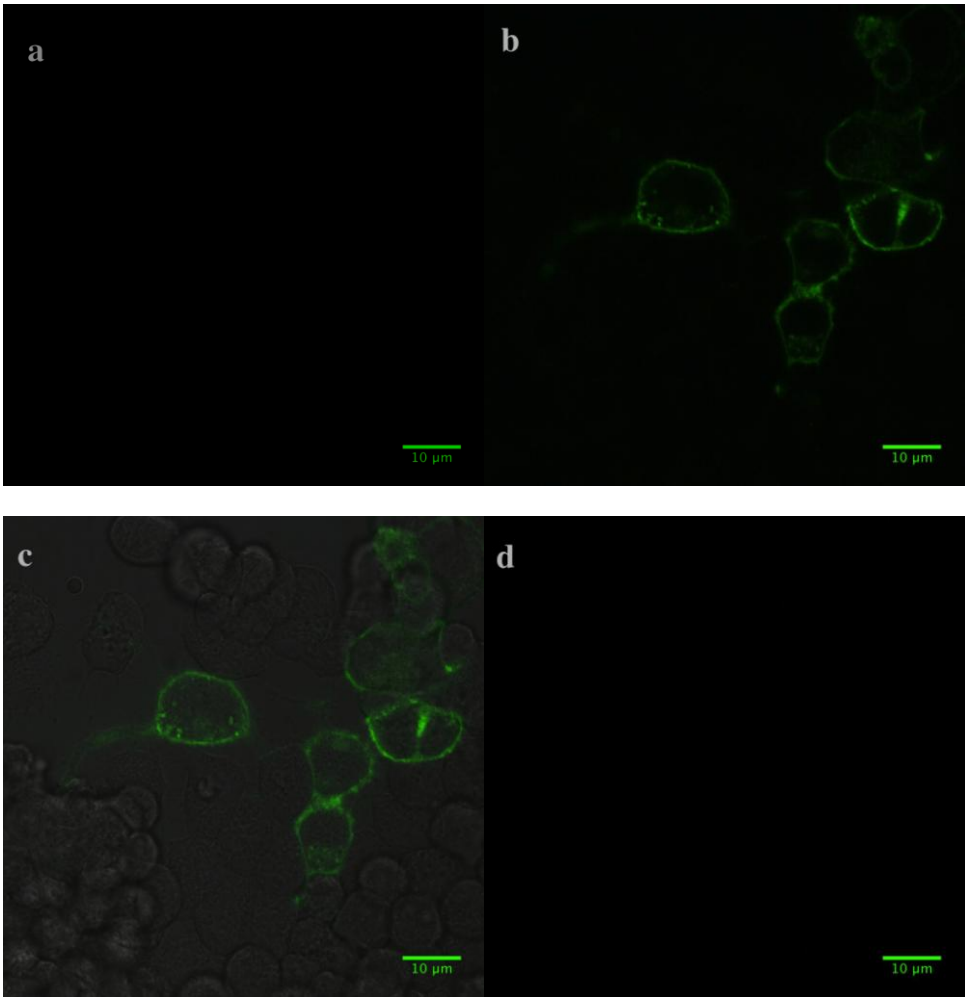

Figure 3.3.1 Confocal images of N2a cells transfected with 200 ng of A2AR _EGFP observed with a) FRET channel b) EGFP channel and c) overlay of EGFP channel and channel D d) mCherry channel (63X oil objective)

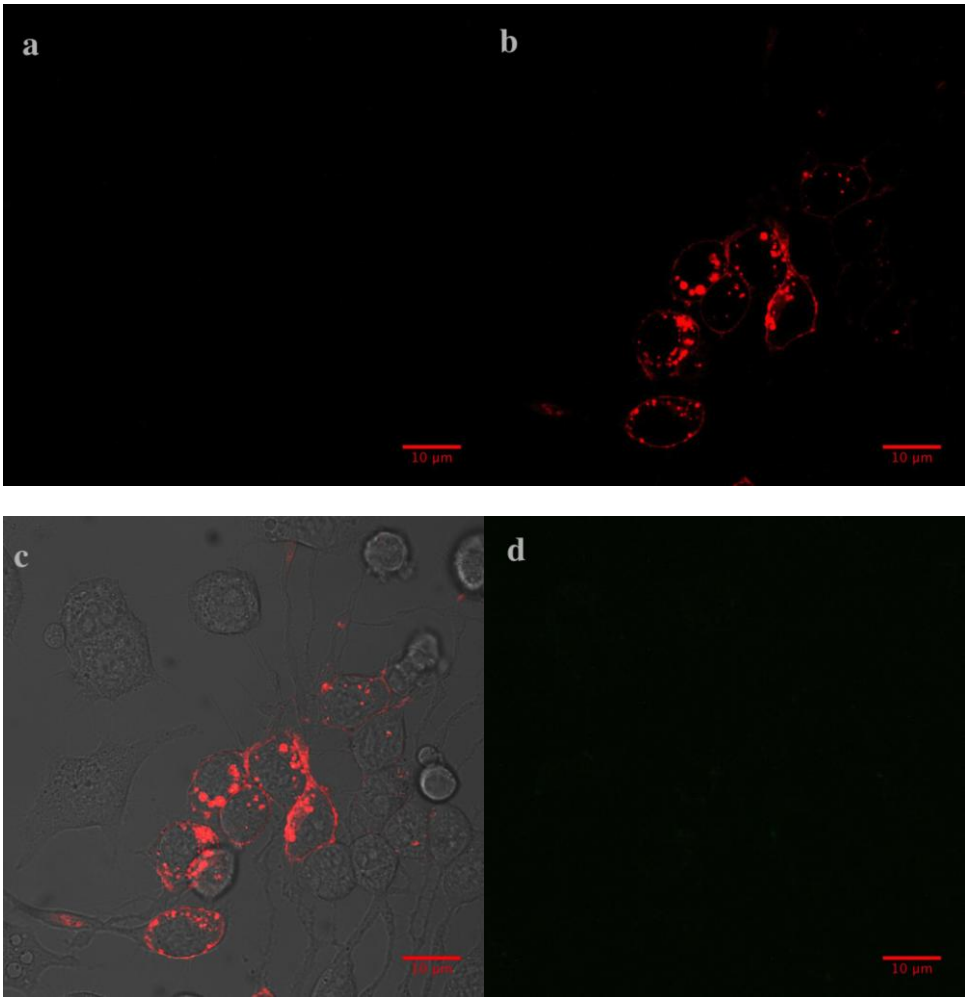

Figure 3.3.2 Confocal images of N2a cells transfected with 200 ng of A2AR _mCherry observed with a) FRET channel b) mCherry channel and c) overlay of mCherry channel and channel D d) EGFP channel (63X oil objective)

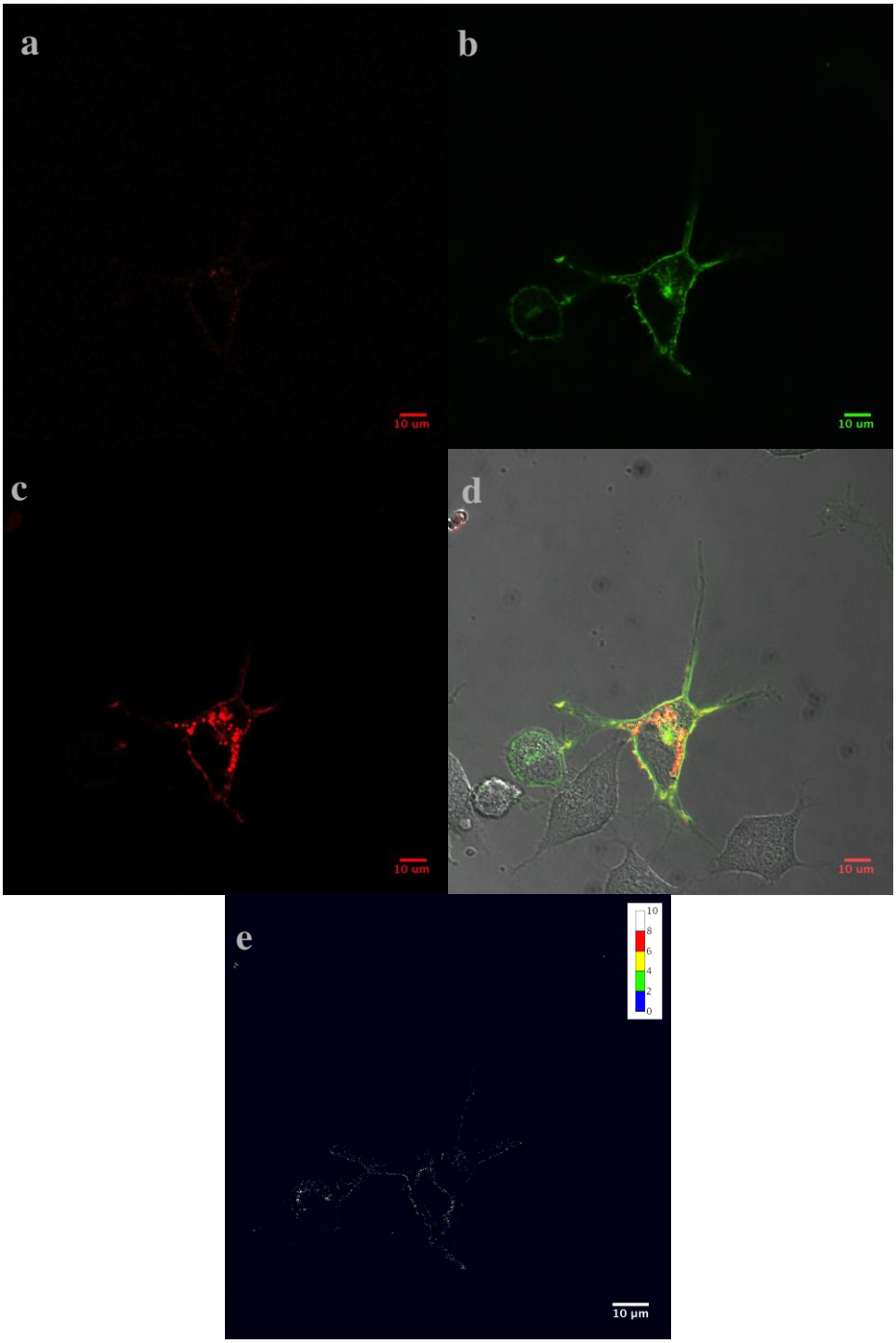

Figure 3.3.3 Confocal images of N2a cells transfected with 200 ng of A2AR_mCherry, and A2AR_EGFP, observed with a) FRET channel, b) EGFP channel, c) mCherry channel, d) overlay of all stacks and channel D e) shows the pixels where FRET efficiency was calculated (63X oil objective)

Consequently, plasma membrane appeared dominantly decorated by both tagged receptors, A2AR-EGFP and A2AR-mcherry transfected in cells (Fig. 3.3.3) and also confocal images indicated the co-localization of A2A receptors occurs mainly on the membrane (Fig. 3.3.3d). In contrast to the co-localization of A2AR-EGFP and A2AR-mcherry in the plasma membrane, FRET signals at either membrane or intracellular parts of cells were detectable (however calculated FRET efficiency was less than 5 %, thus was not suitable for quantitative analysis). There may be several reasons why low FRET efficiency was obtained. Firstly, co-localization of two proteins does not always mean the two proteins are interacting. On the contrary even though two proteins are interacting with each other, we may not observe FRET between them since distance between the proteins are not suitable for the FRET to occur. Secondly, FRET can be affected by the stoichiometry or orientation of acceptors to donors, which can vary with even transfection efficiencies of plasmids. In this thesis, the position of fluorescent protein added to receptors is C-terminal tail and obviously, the orientation of EGFP and mCherry at this position may not allow detectable FRET to occur between these proteins. The important point in FRET studies is to catch the exact time for the location of receptors and the interaction between them. It was quite hard to catch the optimum time for imaging for us since the transportation of samples was required for the live cell imaging of our samples.

3.3.2 Homodimerization of Dopamine D2 Receptors

Similar to Adenosine A2A receptors, the homodimer formation between dopamine D2 receptors has been observed (Ng *et al.*, 1996). Particularly, C-terminally tagged dopamine D2 receptors with split Renilla luciferase 8 have co-expressed in efficient complementation of luminescence (Loening, Fenn, Wu, & Gambhir, 2006). Additionally, heterodimerization studies, especially FRET or BRET studies, have included C-terminally tagged dopamine D2 receptors (Canals, Marcellino, *et al*., 2003). Depending on these studies, C-terminally EGFP and mCherry tagged D2 receptors –have been prepared.

FRET sets were prepared for D2R homodimerization in the same manner as the A2A receptors explained previously. Briefly these sets included three conditions, double transfected cells (EGFP and mCherry tagged receptors) as FRET model, only EGFP tagged receptor transfected cells as donor model and only mCherry tagged receptor transfected cells as acceptor model. In Fig. 3.3.4 and 3.3.5, the images used for donor and acceptor bleed through signals, respectively, were given in different channels. Additionally, Fig. 3.3.6 indicates the representative images for FRET in D2R dimerization studies.

As indicated in images, C- terminally tagged dopamine D2 receptors seemed to be predominantly resided at intracellular compartments. Especially, in FRET images, it has been realized that the co-localization of these receptors is strongly in the intracellular compartments. Due to intercellular retention, FRET efficiencies at plasma membrane are very weak (Fig. 3.3.6e). Although, Guo and his colleagues have shown that C- terminally tagged receptors have expressed in the membrane of live cells (Guo *et al*., 2008), some studies including double labeling experiments indicate that dopamine D2 receptors are mostly found in intracellular compartments. Particularly, long isoform of the dopamine D2 receptors (which we used in this study) are mostly retained in the early secretory membranes and their distribution is essentially restrained in the endoplasmic reticulum (Prou *et al.*, 2001). In this study dopamine D2 receptors showed similar distribution in cells as reported for the long isoform of dopamine D2 receptors reported by Prou *et al*.. On the other hand, according to the confocal images, D2 receptors were distributed not only in intracellular compartments but also plasma membrane that is plasma membrane has been relatively decorated less than inside of the cell.

There are some observations that can provide the retention of Dopamine D2 receptors in intracellular

compartments. First of all, this is not cell – specific and not changing with time that is not delayed transportation (Prou et al., 2001). Secondly, the failures in the folding process of proteins cause some defect in some posttranslational modifications such as glycosylation. Theoretically, it could lead the retrieval of the protein from Golgi complex (Gahmberg & Tolvanen, 1996). In the case of the dopamine D2 receptors, an alternative cause can be that a defect in transport out of ER could lead a mistake in the maturation process of receptors in polysaccharide moieties as mentioned in previous studies (Fishburn, Elazar, & Fuchs, 1995). Finally, the lack of a component in transfected cells that would be involved in the maintenance of the receptors at the plasma membrane and also a lack of a scaffolding proteins such as PDZ-domain proteins, but a consensus for PDZ binding is not defined for the D2 receptor which could have help the explanation of the poor localization of dopamine D2 receptors at the plasma membrane.

From a different perspective, although EGFP and mCherry tagged dopamine D2 receptors –were predominantly co-localized in the intracellular comportments, FRET efficiencies in these compartments are very low (Fig 3.3.6e). The reasons can be same as detailed in section 3.3.1, Homodimerization of Adenosine A2A receptors. Besides to in vivo complications, technical obstacles such as laser sources, incubation of live cells and imaging processes affect the interpretation of FRET signals. In particular, the incubation and transportation of transfected N2a cells used in this study could lead some morphological trouble in the cells. During the imaging process with confocal microscopy, mainly, the vesicular compartments in a cell were observed. These vesicular structures may cause the accumulation of receptors in intracellular compartments, since many signals intensified inside cells. As a conclusion, the health of N2a cells was not good in this thesis.

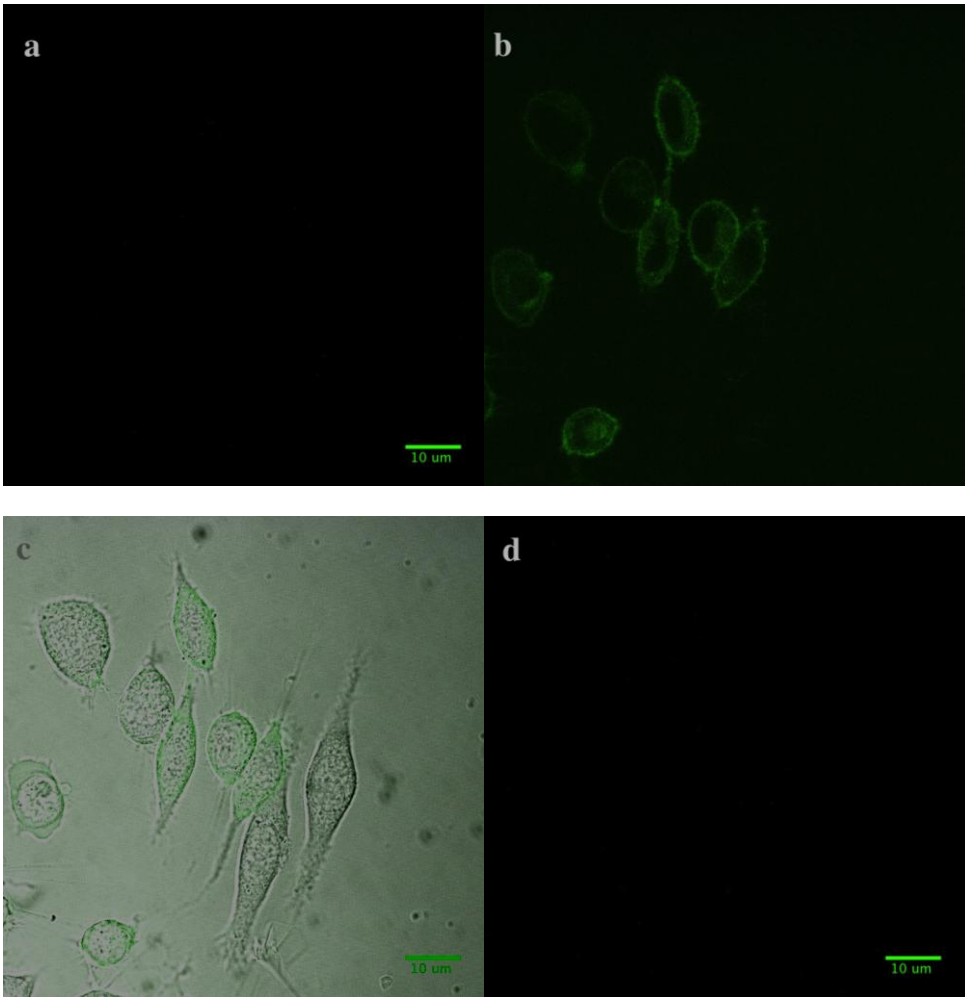

Figure 3.3.4 Confocal images of N2a cells transfected with 200 ng of D2R _EGFP, which is excited at a) FRET channel b) EGFP channel and c) overlay of EGFP channel and channel D d) mCherry channel (63X oil objective)

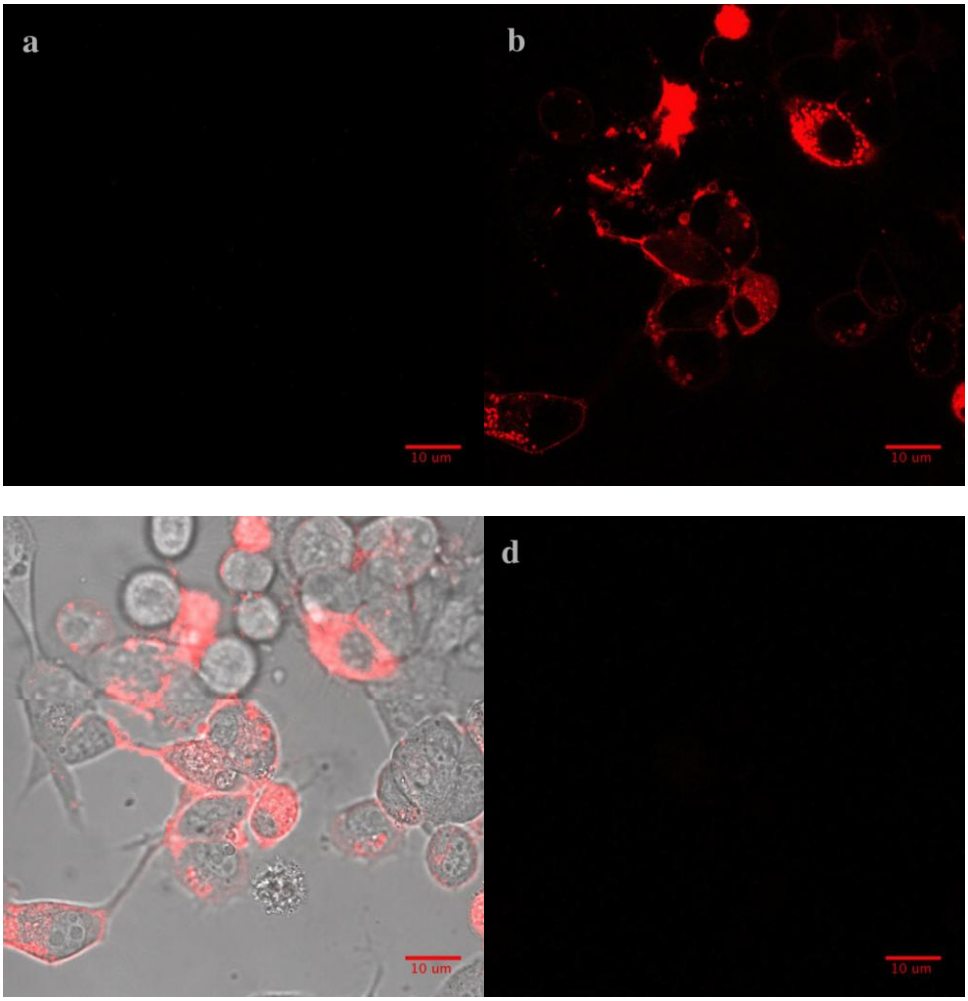

Figure 3.3.5 Confocal images of N2a cells transfected with 200 ng of D2R _mCherry , which is excited at a) FRET channel b) mCherry channel and c) overlay of mcherry channel and channel D d) EGFP channel (63X oil objective)

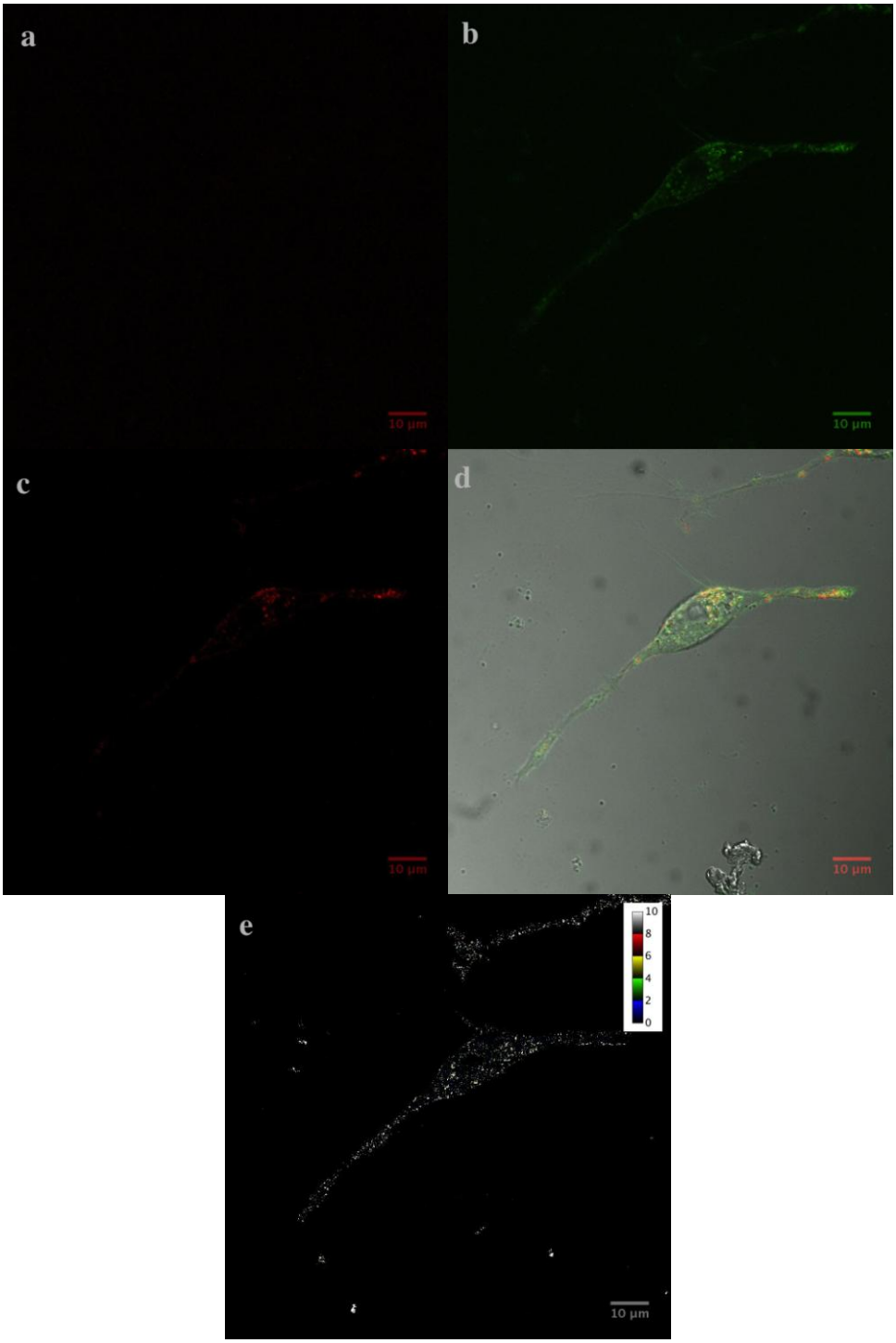

Figure 3.3.6 Confocal images of N2a cells transfected with 200 ng of D2R _mCherry and D2R_ EGFP, which is excited at a) FRET channel, b) EGFP channel, c) mCherry channel, d) overlay of all stacks and channel D e) shows the pixels where FRET efficiency was calculated (63X oil objective)

3.3.3. Heterodimerization of Adenosine A2A and Dopamine D2 receptors

To detect the heterodimerization between A2AR and D2R via FRET, C-terminally tagged receptors – EGFP and mCherry were transfected into N2a cell lines. Some studies such as Canals and his colleagues have shown that functional C-terminally tagged receptors interact with each other on the plasma membrane of HEK293T cell lines (Canals, Marcellino, *et al.*, 2003). In this study, reciprocal FRET samples were prepared to observe FRET between these proteins.

Similar to adenosine A2A and D2 receptors, FRET sets were prepared for heterodimerization included three models, double transfected cells (EGFP and mCherry tagged receptors) as FRET model, only EGFP tagged receptor transfected cells as donor model and only mCherry tagged receptor transfected cells as acceptor model and also the samples were prepared for reciprocal pairs. Similar to homodimerization studies, same images were used for donor and acceptor bleed through signals. Additionally, Fig. 3.3.7 and 3.3.8 indicate the representative images for FRET in heterodimerization studies.

As indicated in figures of this section, both FRET efficiencies in plasma membrane and those in the compartments in the cells are lower or weaker than that in homodimerization of these receptors. Both constructs, A2AR / D2R –EGFP and mCherry, used in this thesis were tagged at C-terminal of receptors. On the other hand, some studies have indicated that that the C-terminal tail of A2AR can be involved in heterodimerization of A2A and D2 receptors (Canals, Burgueño, *et al*., 2003). Although the receptors were co-localizatized, the orientation of acceptor and donor or these proteins could not be suitable for energy transfer. Moreover, some studies indicate that homodimers of adenosine A2A receptors are more favored than heterodimers formed by A2AR and D2R. If the dopamine D2 receptor molecules were not enough to interact with A2A receptors, A2AR transfected into cells may have been inclined to become homodimers. As mentioned before, cellular localization of receptors and technical complications could directly affect FRET studies in this thesis.

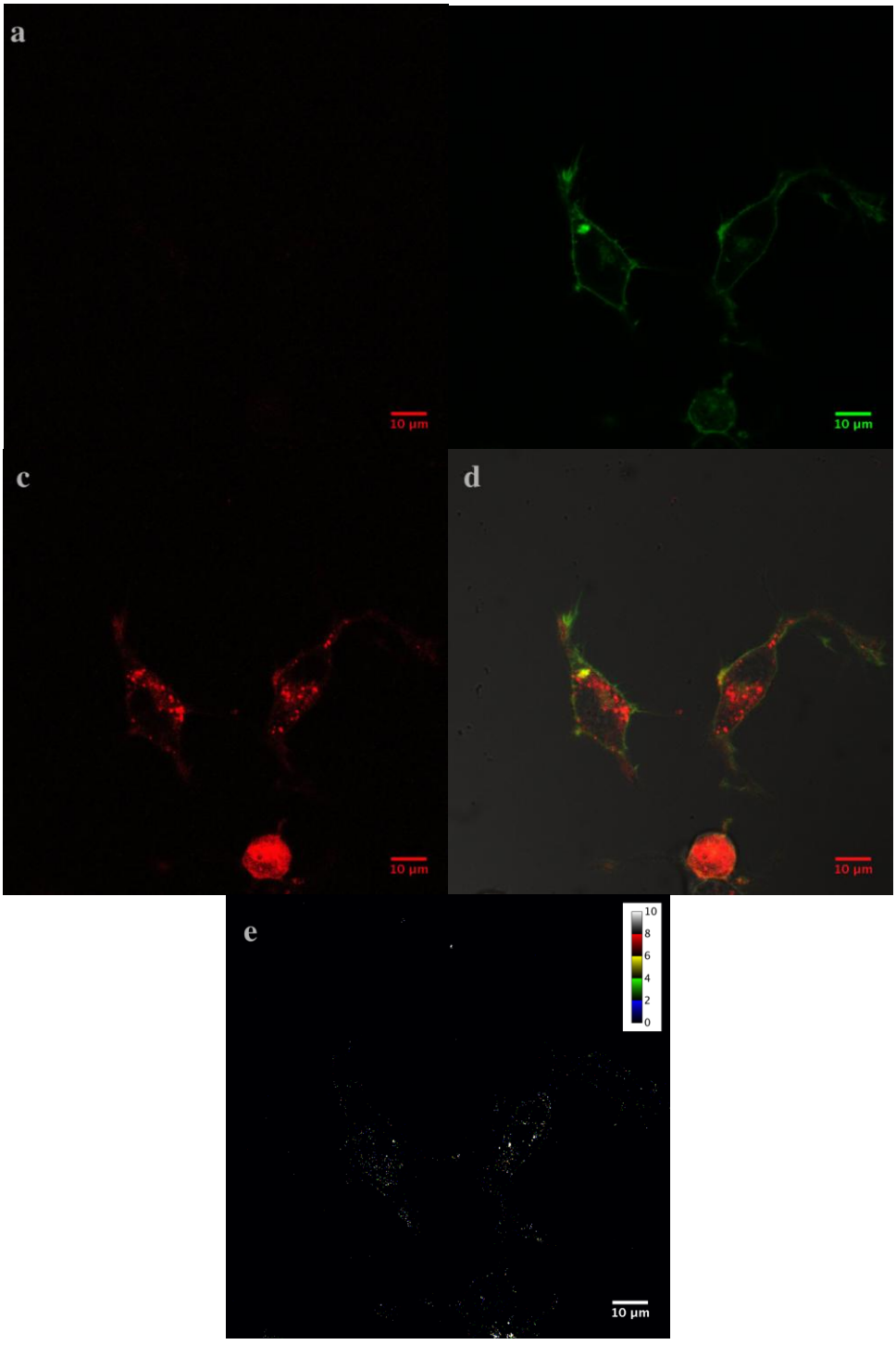

Figure 3.3.7 Confocal images of N2a cells transfected with 200 ng of D2R _mCherry and A2AR_ EGFP, which is excited at a) FRET channel, b) EGFP channel, c) mCherry channel, d) overlay of all stacks and channel D e) shows the pixels where FRET efficiency was calculated (63X oil objective)

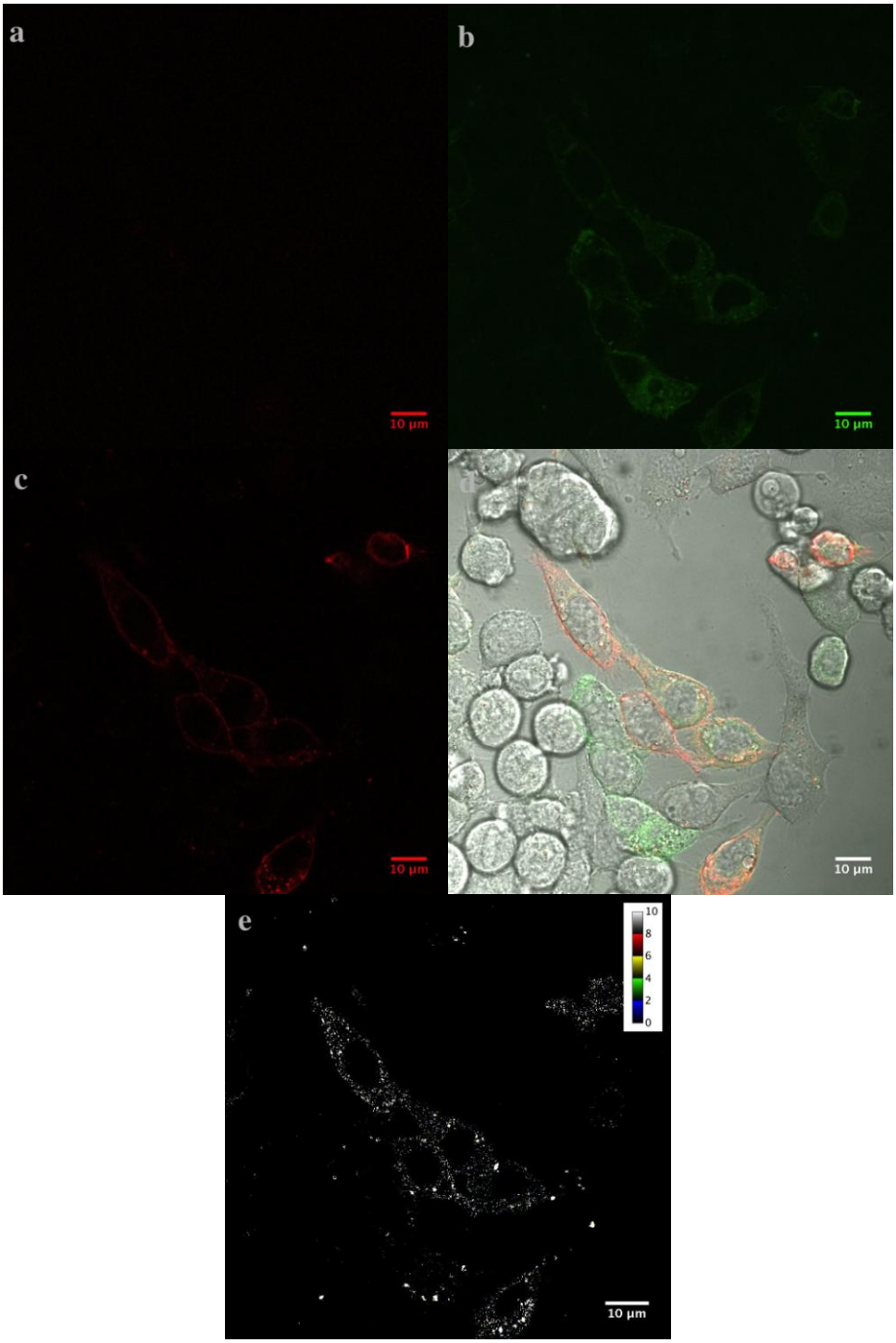

Figure 3.3.8 Confocal images of N2a cells transfected with 200 ng of A2AR _mCherry and D2R_ EGFP, which is excited at a) FRET channel, b) EGFP channel, c) mCherry channel, d) overlay of all stacks and channel D e) shows the pixels where FRET efficiency was calculated (63X oil objective)

As a consequence, functional C-terminally tagged A2AR and D2R constructs were used to detect heterodimerization of these receptors, as well homodimerization of them. The colocalization of the receptors indicates that the expressions of both receptors were found at both cellular membrane and intracellular compartments. However, as mentioned before, intracellular retention of dopamine D2 receptors and tagging position of receptors could lead the inconvenient environment to occur FRET at membrane. Therefore, in agreement with colocalization of receptors, these receptors coexpressed at plasma membrane for interaction; however, FRET signals are not enough to calculate the distance between these receptors.

CHAPTER 4

CONCLUSION

The aims of the study were to assess the functionality of fluorescently tagged adenosine A2A and dopamine D2 receptors expressed in N2a mouse neuroblastoma cell line and to quantitatively assess the interaction of these two receptors as homodimerization and heterodimerization in live cells.

At the end of the study, the functionality of C- terminally tagged receptors was assessed. According to their ability to increase or reduce cAMP production in cells, the functionality of the fusion receptors was confirmed. Homodimerization of adenosine A2A and dopamine D2 receptors were presented, as well heterodimerization of A2AR-D2R by using the configurations of FRET in confocal laser scanning microscope. However, to obtain quantitative FRET signals, the position of fluorescent molecule could be changed, to optimize fluorophore interaction.

Using FRET method, possible drugs targeted to neurophysiological disorders, dopamine and adenosine agonists/antagonists were tested. Unfortunately, no significant change in FRET was observed. The concentration and incubation time should be optimized for future studies.

With this thesis study, a powerful, fluorescence based cell culture model to detect the homo- and hetero-dimerization of adenosine A2A and dopamine D2 receptors has been successfully developed. This model system is readily usable for drug screening research. Drugs that affect dopamine or adenosine signaling can be introduced to this system and their effect on homo- and hetero-dimerization of these receptors can be investigated very easily. Thus, this system is also useful to study/screen molecular mechanisms of some chemicals as potential drugs.

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APPENDIX A

METABOLIC REACTIONS

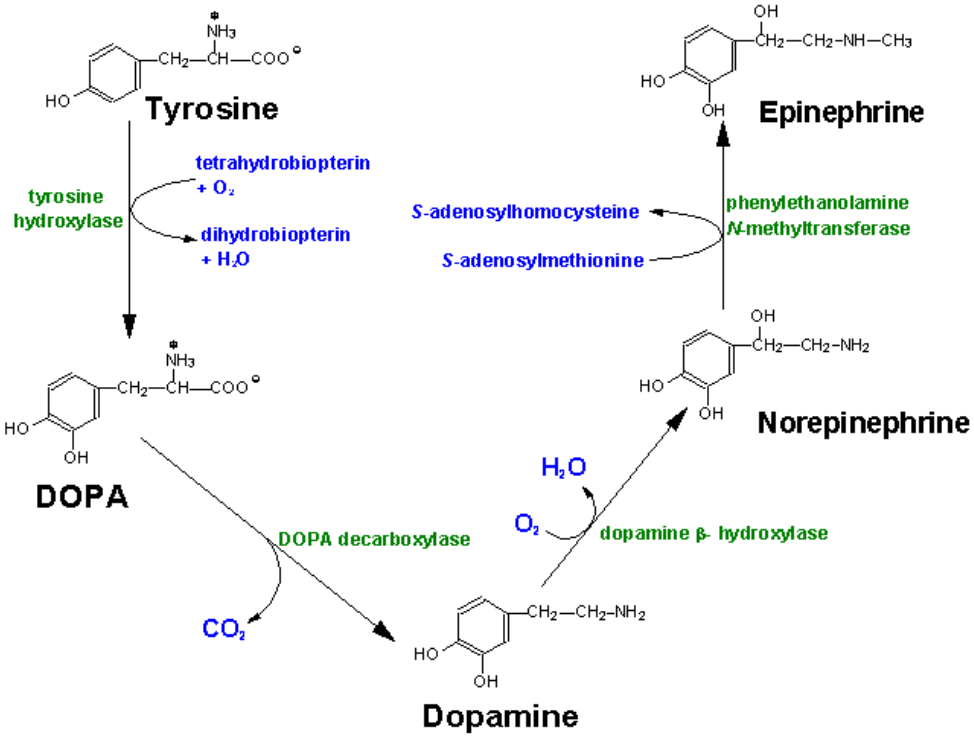

Figure A.1 Metabolic reactions of dopamine (adapted from http://www.bio.davidson.edu)

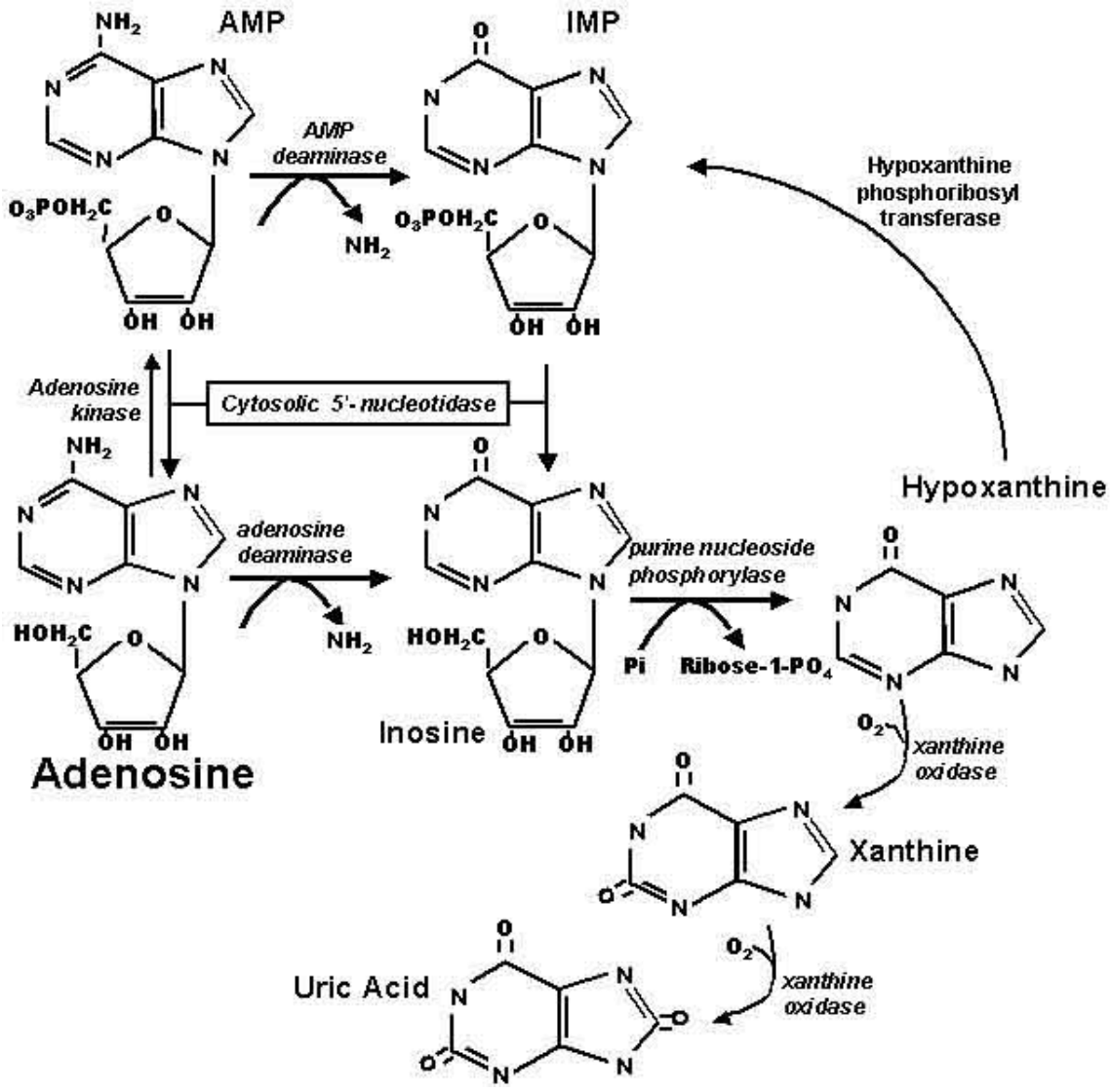

Figure A.2 Intracellular metabolism of adenosine (Conlon, et al., 2005)

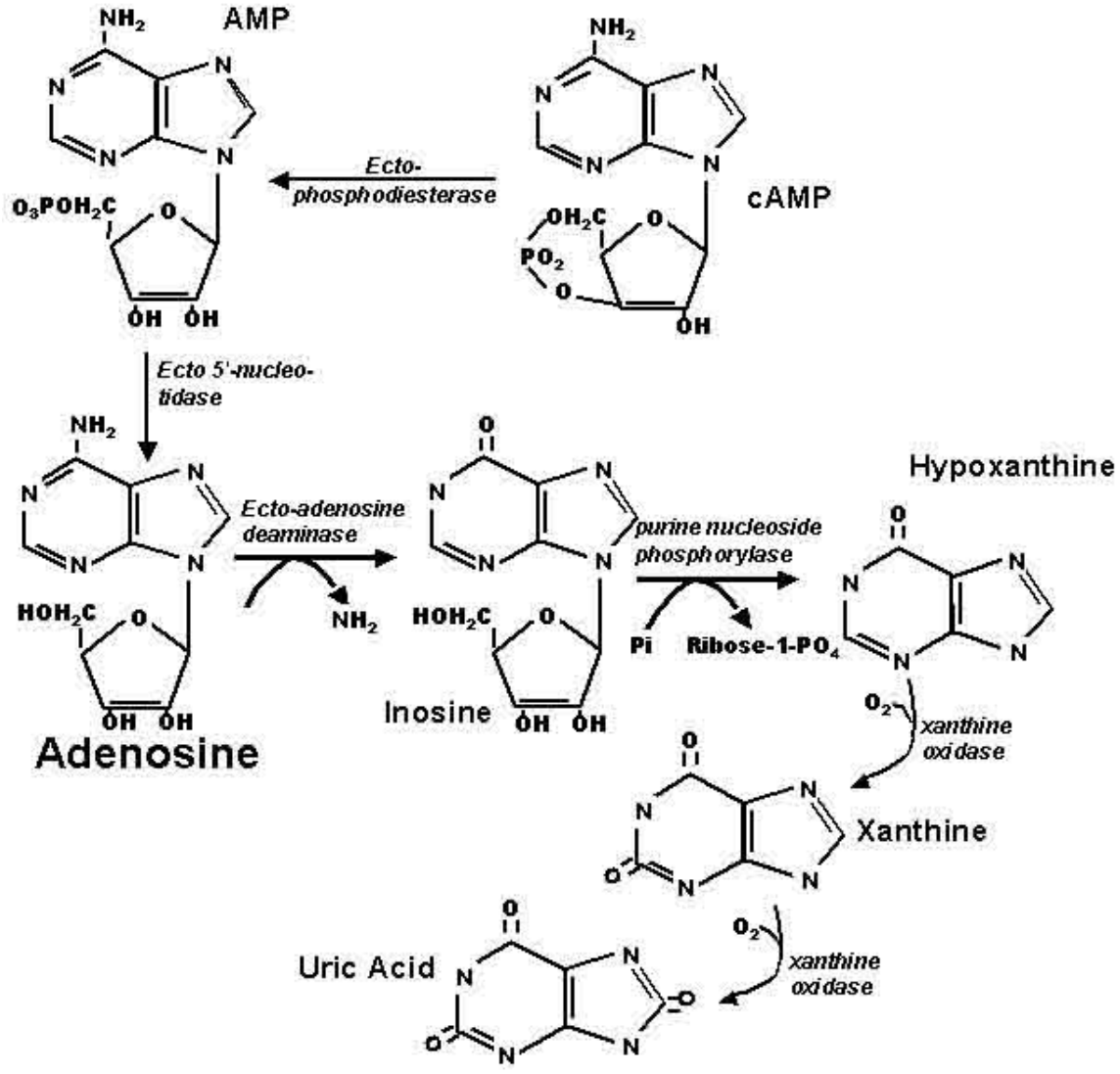

Figure A.3 Extracellular metabolism of adenosine (Conlon, et al., 2005)

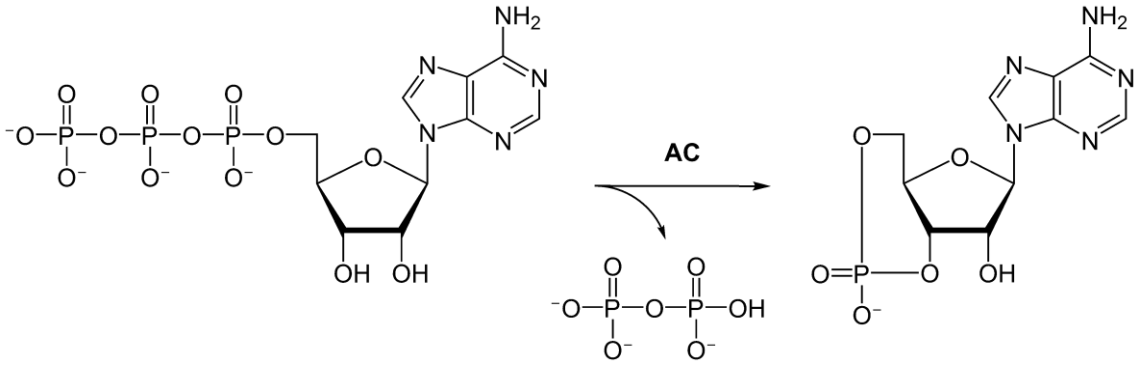

Figure A.4 Conversion of ATP to cAMP by the action of adenylyl cyclase

CHEMICAL STRUCTURES OF AGONISTS

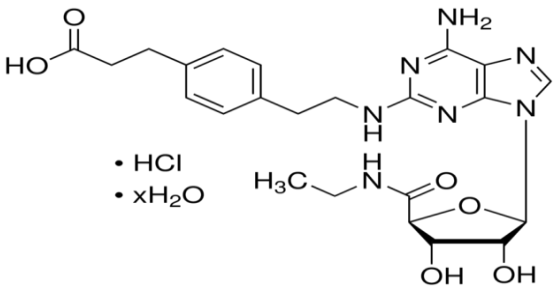

Figure A.5 Chemical structure of CGS21680, a selective antagonist of A_{2A} receptor (taken from Sigma Aldrich Co.)

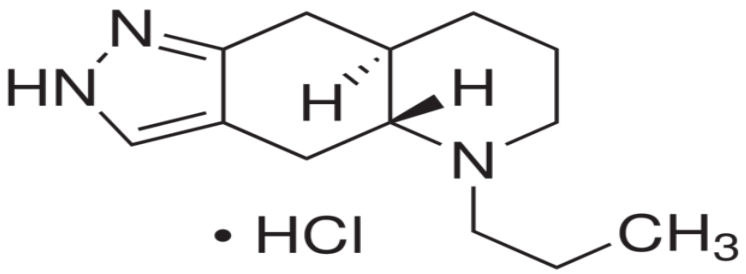

Figure A.6 Chemical structure of Quinpirole, a selective agonist of D2 receptor (taken from Sigma Aldrich Co.)

APPENDIX B

NEURO2A CELL CULTURE MEDIUM

Table B.1 Composition of D-MEM with High Glucose

COMPONENT	CONCENTRATION (mg/L)
Amino acids	
Glycine	30
L-Arginine hydrochloride	84
L-Cysteine 2HCl	63
L-Glutamine	580
L-Histidine hydrochloride-H2O	42
L-Isoleucine	105
L-Leucine	105
L-Lysine hydrochloride	146
L-Methionine	30
L-Phenylalanine	66
L-Serine	42
L-Threonine	95
L-Tryptophan	16
L-Tyrosine	72
L-Valine	94
Vitamins	
Choline chloride	4

Table B.1 cont'd Composition of D-MEM with High Glucose

D-Calcium pantothenate	4
Folic acid	4
Niacinamide	4
Pyridoxine hydrochloride	4
Riboflavin	0.4
Thiamine hydrochloride	4
i-Inositol	7.2
Inorganic Salts	
Calcium chloride	264
Ferric nitrate	0.1
Magnesium sulfate	200
Potassium chloride	400
Sodium bicarbonate	3700
Sodium chloride	6400
Sodium phosphate monobasic	141
Other components	
D-Glucose (Dextrose)	4500
Phenol Red	15
Sodium pyruvate	110

APPENDIX C

BACTERIAL CULTURE MEDIA PREPARATION

Luria- Bertani (LB) Medium
10 g/L tryptone
5 g /L yeast extract
5 g/L NaCl

15 g/L agar is added to LB for solid agar medium preparation.

APPENDIX D

BUFFERS AND SOLUTIONS

10X TBE (Tris-Borate-EDTA) Buffer

Component	Amount	Concentration
Tris Base	108 g	890 mM
Boric Acid	55 g	890 mM
EDTA	40 ml	20 mM

For 1X TBE this solution was diluted 1:10 and used for electrophoresis.

Composition of 6X Loading Dye
10 mM Tris-HCl (pH 7.6)
0.03% bromophenol blue
0.03% xylene cyanol FF
60% glycerol
60 mM EDTA
Transformation Buffers
Buffers

Transformation buffer I (TfbI):

30 mM KOAc
100 mM RbCl2
10 mM RbCl2
50 mM MnCl2
15% Glycerol (v/v)
pH = 5.8

Transformation buffer II (TfbII):

10 mM MOPS (or PIPES)
75 mM CaCl2
10 mM RbCl2
15% Glycerol (v/v)
pH = 6.5

10X PBS (Phosphate Buffered Saline) Buffer
80g NaCl
2g KCl
7.62g Na2HPO4
0.77g KH2PO4
pH=7.4
1X Induction Buffer
500µM isobutyl-1-methylxanthine (IBMX)
100µM 4-(3-butoxy-4-methoxy-benzyl) imidazolidone (Ro 20-1724)
Components were dissolved in 1X PBS buffer.

APPENDIX E

PLASMID MAPS

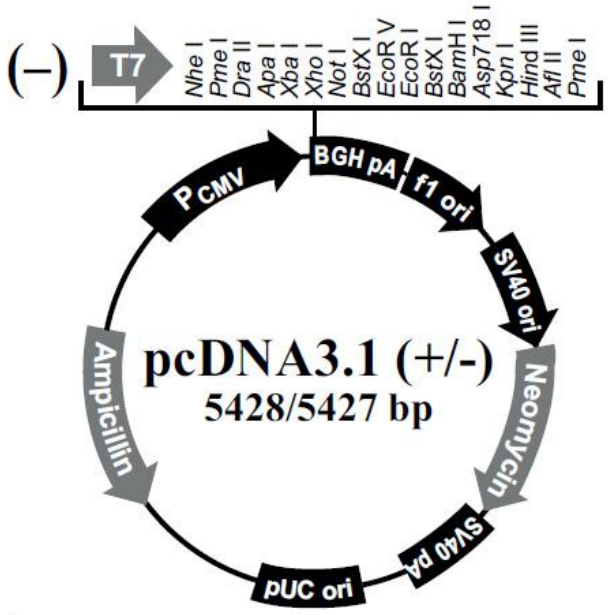

Figure E.1 Map of pcDNA 3.1 (-) (image taken from Invitrogen® Life Technologies)

SEQUENCES OF FLUORESCENT PROTEIN TAGGED RECEPTORS

ATGCCCATCATGGGCTCCTCGGTGTACATCACGGTGGAGCTGGCCATTGCTGTGCTGGCCATCCTGGGCAATGTGC
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GTCCATGCTGCCAAGTCACTGGCCATCATTGTGGGGCTCTTTGCCCTCTGCTGGCTGCCCCTACACATCATCAACT
GCTTCACTTTCTTCTGCCCCGACTGCAGCCACGCCCCTCTCTGGCTCATGTACCTGGCCATCGTCCTCTCCCACAC
CAATTCGGTTGTGAATCCCTTCATCTACGCCTACCGTATCCGCGAGTTCCGCCAGACCTTCCGCAAGATCATTCGC
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CCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA

Figure E.2 Coding sequence of A2AR+mCherry fusion. Black sequence belongs to A2AR (Accession Number: NM_000675), red sequence belongs to mCherry coding sequences (Accession Number: ACO48282).

ATGGATCCACTGAATCTGTCCTGGTATGATGATGATCTGGAGAGGCAGAACTGGAGCCGGCCCTTCAACGGGTCAGA
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CAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGG
GCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA

Figure E.3 Coding sequence of D2R+mCherry fusion gene. Black bases belong to D2R coding sequence (Accession Number: NM_000795); red bases belong to mCherry cDNA (Accession Number: ACO48282)

ATGCCCATCATGGGCTCCTCGGTGTACATCACGGTGGAGCTGGCCATTGCTGTGCTGGCCATCCTGGGCAATGTGCT
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CACTCTCGGCATGGACGAGCTGTACAAGTAA

Figure E.4 Coding sequence of A2AR+EGFP fusion gene. Black bases belong to A2AR coding sequence (Accession Number: NM_000675); green bases belong to EGFP cDNA (Accession Number: AAB02574)

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GCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAG
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CCACTACCTGAGCACCCAGTCCAAGCTTAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCG
TGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA

Figure E.5 Coding sequence of D2R+EGFP fusion gene. Black bases belong to D2R coding sequence (Accession Number: NM_000795); green bases belong to EGFP cDNA sequence (Accession Number: AAB02574)

APPENDIX F

PRIMERS

Primers to amplify mCherry cDNA with A2AR flanking ends

Forward primer for mCherry-A2AR:
5’ - cccctggcccaggatggagcaggagtgtccATGGTGAGCAAGGGCGAGGAG - 3’
Reverse primer for mCherry-A2AR:
5’ - cgcgccaaacgaatggtctagaaagcttccTTACTTGTACAGCTCGTCCATGCC - 3’

Small case bases at the 5’ end of the forward primer belong to the last 30 bp of A2AR excluding stop codon. On the other hand, small case bases at the reverse primer correspond to the 30 bases on the pDNR-Dual vector, which directly follow A2AR sequence. Capital letters show the primer sequences that anneal to mCherry gene.

Prmers to amplify EGFP/mCherry with D2R flanking regions

Forward primer for EGFP/mCherry-D2R:
5’ – cgcaaggccttcctgaagatcctccactgcATGGTGAGCAAGGGCGAGGAG – 3’
Reverse primer for EGFP/mCherry-D2R:
5’ – ctgatcagcggtttaaacttaagcttccTTACTTGTACAGCTCGTCCATGCC – 3’

Small case letters at the 5’ end of the forward primer are the last 30 bp of D2R and those at the 5’ are 30 bp of the pcDNA 3.1 (-) following D2R cDNA sequence. Capital letters are the parts that anneal to both EGFP and mCherry genes since these sequences are shared by both genes.

Primers to amplify EGFP with A2AR flanking regions

Forward primer for EGFP-A2AR
cccctggcccaggatggagcaggagtgtccttgATGGTGAGCAAGGGCGAGGAG
Reverse primer for mCherry-A2AR
ctgatcagcggtttaaacttaagcttccTTACTTGTACAGCTCGTCCATGCC

Small case letters at the 5’ end of the forward primer are the last 30 bp of A2AR and those at the 5’ are 30 bp of the pcDNA 3.1 (-) following D2R cDNA.