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Noncanonical Neurotransmitter Activation of Catecholamine Receptors

Marta Sánchez Soto

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UNIVERSITAT DE BARCELONA

FACULTAT DE BIOLOGIA

DEPARTAMENT DE BIOQUÍMICA I BIOMEDICINA MOLECULAR

NONCANONICAL NEUROTRANSMITTER ACTIVATION OF CATECHOLAMINE RECEPTORS

Marta Sánchez Soto

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NONCANONICAL NEUROTRANSMITTER ACTIVATION OF CATECHOLAMINE RECEPTORS

Memòria presentada per la llicenciada en Biologia

MARTA SÁNCHEZ SOTO

per a optar al grau de Doctor per la Universitat de Barcelona

El treball experimental i la redacció de la present memòria han estat realitzats per Marta Sánchez sota la direcció dels Dr. Sergi Ferré i el Dr. Vicent Casadó.

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LIST OF ABBREVIATIONS

5-HT: 5-hydroxytryptamine or serotonin

7TM: seven transmembrane domain

α2AR: alpha$_{2A}$-adrenoceptor

α2CR: alpha$_{2C}$-adrenoceptor

AC: adenylyl cyclase

A2AR: adenosine A_{2A} receptor

ADHD: attention-deficit hyperactivity disorder

BiFC: bimolecular fluorescence complementation

BRET: bioluminescent resonance energy transfer

cAMP: cyclic adenosine monophosphtate

CODA-RET: complemented donor-acceptor resonance energy transfer

DA: dopamine

D1R: dopamine D_1 receptor

D2R: dopamine D_2 receptor

D3R: dopamine D_3 receptor

D4R: dopamine D_4 receptor

D5R: dopamine D_5 receptor

DAT: dopamine transporter

DβH: dopamine β-hydroxylase

ECL: extracellular loop

ERK: extracellular signal-regulated kinase

FACS: fluorescence-activated cell sorting

FCS: fluorescence correlation spectroscopy

FRET: fluorescent resonance energy transfer

GABA: gamma-aminobutyric acid

GDP: guanosine diphosphate

GIRK: G protein-coupled inwardly-rectifying potassium channels

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

GTP: guanosine triphosphate

HEK: human embryonic kidney

ICL: intracellular loop

LC: locus coeruleus

MAO: monoamine oxidase enzyme

MAPK: mitogen-activated protein kinase

MSN: medium spiny neuron

NE: norepinephrine

NET: norepinephrine transporter

PD: Parkinson's disease

PI3K: phosphatidylinositol 3-kinase

PKA and PKC: protein kinase A and C

PLC: phospholipase C

RET: resonance energy transfer

RGS: regulators of G protein signaling

RLS: restless leg syndrome

Rluc: Renilla luciferase

SNC: central nervous system

SUD: substance use disorders

TH: tyrosine hydroxylase

TM: transmembrane

TR: tandem repeats

VMAT: vesicular monoamine transporter

VNTR: variable number of tandem repeats

YFP: yellow fluorescent protein

I. INTRODUCTION

I. INTRODUCTION

1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) or seven transmembrane domain receptors (7TM) comprise the largest superfamily of proteins in the body. In humans, more than 2% of the genome codes for 700-800 GPCRs (Jacoby et al., 2006), 90% of which are expressed in the Central Nervous System (SNC) (Gudermann et al., 1997; George et al., 2002). GPCRs are known as extremely versatile receptors for extracellular messengers as diverse as biogenic amines, purines and nucleic acid derivatives, lipids, peptides and proteins, odorants, pheromones, tastants, ions like calcium and protons, and even photons in the case of rhodopsin (Jacoby et al., 2006). GPCRs are targets for approximately 40% of pharmacological therapeutics (Garland, 2013; Cvicek et al., 2016) and provide further important opportunities for the development of new drug candidates with potential applications in all clinical fields.

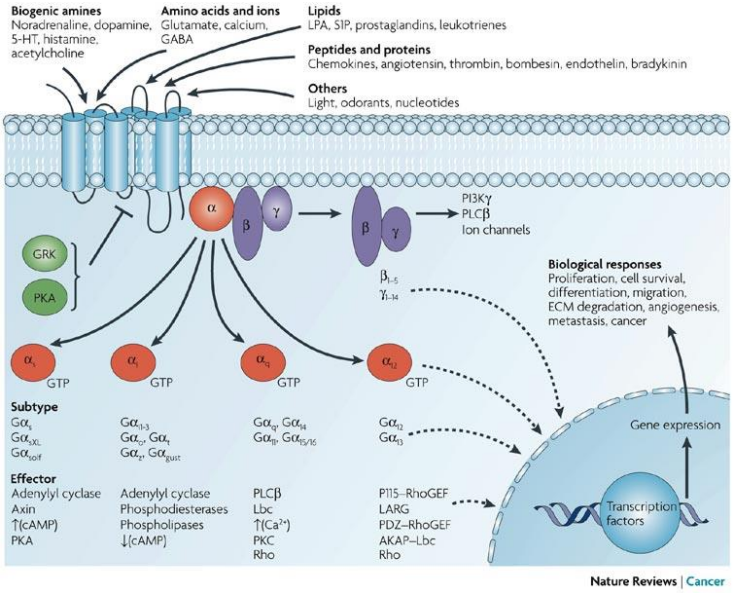

Figure 1. Diversity of G protein-coupled receptor signaling. A wide variety of ligands use GPCRs to stimulate membrane, cytoplasmic and nuclear targets through heterotrimeric G protein-dependent and -independent pathways. Such signaling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. The aberrant activity of G proteins and their downstream target molecules can contribute to cancer progression, metastasis and other diseases. 5-HT, 5-hydroxytryptamine; ECM, extracellular matrix; GABA, gamma-aminobutyric acid; GEF, guanine nucleotide exchange factor; GRK, G protein receptor kinase; LPA, lysophosphatidic acid; PI3K, phophatidylinositol 3-kinase; PKA and PKC, protein kinase A and C; S1P sphingosine-1-phosphate. *Extracted from Dorsam and Gutkind 2007.*

1.1. Structure of GPCRs

GPCRs are characterized by a seven-transmembrane alpha helical (7TM) configuration with the N-terminal part facing the extracellular side and a carboxyl side facing the interior of the cell. The transmembrane domains are formed by 25 to 35 amino acids with a high degree of hydrophobicity. The first crystal structure of a GPCR appeared in the year 2000, when Palczewski et al. (2000) reported the high resolution structure for the bovine rhodopsine receptor (Fig 2). With a 2.3 Å resolution, it was confirmed that the α-helical transmembrane domains rearranged in a closely packed bundle forming the transmembrane receptor core and they were connected through 3 intracellular and 3 extracellular loops (ICL and ECL, respectively).

In general, GPCRs do not share any overall sequence homology; the only structural feature common to all GPCRs is the presence of the 7TM connected by alternating intracellular and extracellular loops. Two cysteine residues conserved in most GPCRs (one in ECL1 and one in ECL2) form a disulfide bond, which is presumed to be important for the packaging and stabilization of a restricted number of conformations of these seven transmembrane domains (Probst, 1992; Baldwin, 1994). Aside from wide sequence variations, GPCRs differ in their lenght and function of their extracellular N-terminal domain, their intracellular C-terminal domain and the six alternating ICL and ECL loops. Each of these domains provides specific properties to the receptors. However, significant sequence homology is found within several subfamilies of the GPCR superfamily (Attwood and Findlay, 1994).

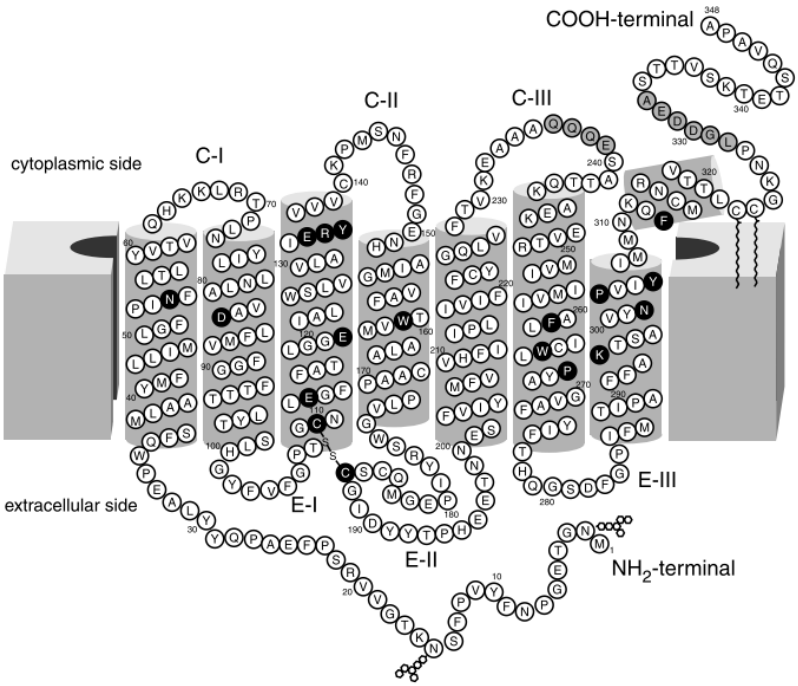

Figure 2. Two-dimensional model of bovine rhodopsin. Some of the key residues are shown in filled circles, while residues not modeled in the current structure are shown in gray circles. *Extracted from Palczewski et al., 2000.*

1.2. Classification of GPCRs

One of the first GPCR classification systems was introduced by Kolakowski for the now defunct GCRDb database (Kolakowski, 1994). GPCRs were divided into seven groups, designated A–F

and O, derived from original standard similarity searches. This system was further developed for the GPCRDB database (Horn *et al.,* 2003), which divides the GPCRs into six classes. The three major families or classes of receptors include those related to the rhodopsin and the β_2-adrenergic receptor (family A), those related to the glucagon receptor (family B), and those related to the metabotropic neurotransmitter receptors (family C). Three minor families include Class D, composed of pheromone receptors, Class E of cAMP receptors and class F formed by the Frizzled/smoothened family.

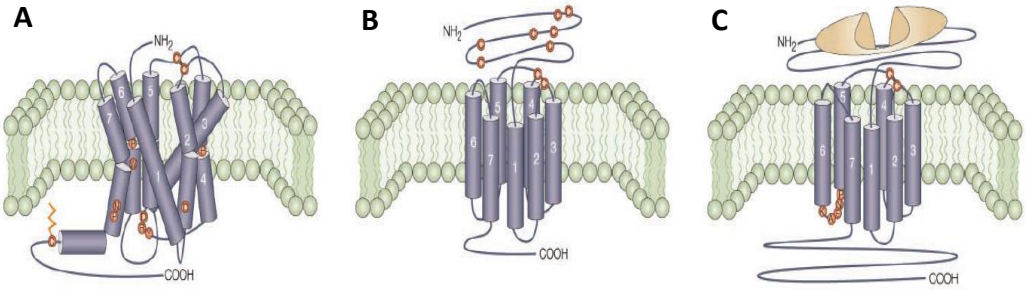

Figure 3. GPCRs classification. GPCRs can be divided into three main families: A, B and C. Highly conserved key residues are indicated in red circles. *Extracted from George et al., 2002.*

Family A receptors, also called rhodopsin-like receptors, comprise the largest and most studied family of GPCRs. This class of receptors binds ligands from various types, including small molecules such as biogenic amines as well as peptides. The overall homology among all type A receptors is low and restricted to a number of highly conserved key amino acid residues. The high degree of conservation among these key residues suggests that they play an essential role on the structural or functional integrity of the receptors. The only residue that is conserved among all family A members is the arginine in the Asp-Arg-Tyr (DRY) motif located in the cytosolic side of the third transmembrane domain (Probst et al., 1992) that is considered to be involved in the G protein activation. Despite limited sequence homology, class A receptors exhibit identical structural organization, and their overall topography can be subdivided into three main regions. On the extracellular side, the N-terminal region is involved in ligand binding and possibly receptor activation, whereas the extracellular loops represent important key elements for peptide binding and play a role in receptor selectivity towards ligands. The transmembrane core is comprised of a bundle of seven alpha-helices

that provide a hydrophobic environment critical for nonpeptide as well as small-peptide ligand binding. It relays the conformational changes induced upon ligand binding on the extracellular side of the receptor to the intracellular architectural determinants that regulate activation of the signaling cascade. On the intracellular side, the loop regions contain key elements for either direct or scaffolding-protein-dependent interactions with intracellular effectors. Additionally, posttranslational modifications present in the C-terminal are likely to modulate both receptor activation state and G protein coupling as well as to participate in the regulation of receptor internalization and desensitization. Many of the Class A receptors have a palmitoylated cysteine on the C-terminal that works as anchorage to the plasma membrane (Papac et al., 1992; Kennedy and Limbird, 1993). To this family belong the receptors studied in this thesis: dopamine and adrenergic receptors.

Family B receptors, also called secretin-like receptors, include about 15 different receptors for various hormones and neuropeptides such as the Vasoactive intestinal peptide (VIP), calcitonine, parathyroid hormone (PTH) or glucagon. Except for the disulfide bridge between ECL1 and ECL2, family B receptors don't have common structures with family A and importantly, they don't have the DRY motif. They share a large extracellular amino terminus with conserved cysteine residues and disulfide bridges (Ulrich et al., 1998) that is important for the ligand recognition (George et al., 2002; Jacoby et al., 2006). Some proline residues are also conserved within the helical bundle, but those residues are different from family A conserved prolines (reviewed in Culhane et al., 2015).

Family C receptors include the metabotropic glutamate, the γ-aminobutyric acid (GABA) and the calcium receptors, among others. Similar to the previous families, these receptors have conserved cysteines forming disulfide bridges between ECL1 and 2. Members of this family have a very large extracellular domain (500-600 amino acids). They are also characterized by having a short ICL3 highly conserved. The ligand binding site of family C is believed to be located within the amino terminus domain (O'Hara et al., 1993; Conn and Pinn, 1997).

There are two further GPCR families that are considerably smaller. Class D is composed of pheromone receptors, which are used by organisms for chemical communication (Nakagawa et al., 2005) while class E, the cAMP receptors, are part of the chemotactic signaling system of slime molds (Prabhu and Eichinger, 2006). There is also an additional minor class, the

Frizzled/Smoothened receptors, which are necessary for Wnt binding and the mediation of hedgehog signaling, a key regulator of animal development (Foord et al., 2002).

Not all human GPCRs can be effectively classified using this system, there are approximately 60 “orphan” GPCRs that show the sequence properties of Family A rhodopsin-like receptor but for which there are not defined ligands or functions (Gloriam et al., 2005).

1.3. Signaling of GPCRs

GPCRs were named for their common ability to associate with heterotrimeric G proteins (GαβϒY). These are the molecular switches that turn on intracellular signaling cascades in response to the activation of GPCRs by extracellular stimuli. Therefore, G proteins have a crucial role in defining the specificity and temporal characteristics of the cellular response. Heterotrimeric G proteins are composed of three subunits, α, β and γ, and their switching function depends on the ability of the G protein α subunit (Gα) to cycle between an inactive GDP-bound conformation that is primed for interaction with an activated receptor, and an active GTP-bound conformation that can modulate the activity of downstream effector proteins.

The classic G protein cycle is shown in Fig 4 and can be summarized as follows: In the absence of agonist activation of the receptor, the α-subunit is bound to GDP and in close formation with the βϒ-complex, which is often referred as the inactive complex. Upon agonist binding, a series of conformational changes within the receptor triggers further conformational changes to the associated heterotrimeric G protein. This event leads to nucleotide exchange (GDP for GTP) to the α subunit producing a dissociation of α and the βϒ complex (Marinissen and Gutkind, 2001). The free α-subunit and βϒ-subunit complex interact and modulate the activity of down-stream elements of the signaling cascades such as adenylyl cyclase (AC), phospholipases, or calcium and potassium ion channels. Besides the regulation of these classical second-messenger generating systems, Gβγ subunits can also control the activity of key intracellular signal-transducing molecules, including small GTP-binding proteins of the Ras and Rho families and members of the mitogen-activated protein kinase (MAPK) family of serine-threonine kinases, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), p38 and ERK5, through an intricate network of signaling events that has yet to be elucidated. Ultimately, the integration of the functional activity of the G protein-regulated signaling networks control many cellular functions and the aberrant activity of G proteins and their downstream target molecules can contribute to cancer progression and other diseases (Dorsam and Gutkind, 2007). When GTP gets hydrolyzed to GDP in the α-subunit, the GDP-bound α-subunit binds again to the βϒ-subunit complex and forms the inactive G protein complex (Hamm, 1998). The hydrolysis of GTP to GDP is mediated by regulators of G protein signaling (RGS).

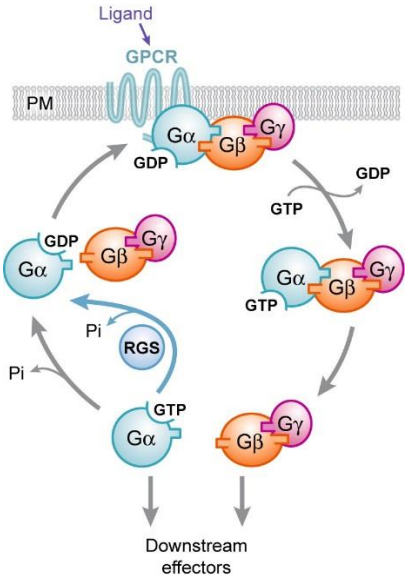

Figure 4. G protein cycle. *Extracted from Li et al. 2007*

The exposure of GPCRs to agonists often results in the rapid attenuation of receptor responsiveness, a process called desensitization. This phenomenon is the consequence of a combination of different mechanisms that include the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation (Hausdorff, 1989; Lohse et al., 1990), the internalization of cell surface receptors to various intracellular compartments (Hermans, 1997; Trejo, 1998), and the down-regulation of the number of receptors in the cell. The latter is accomplished through mechanisms to reduce receptor mRNA and protein synthesis, as well as the lysosomal degradation of pre-existing receptors (Jockers et al., 1999; Pak et al., 1999). Interestingly, it is now well known that agonist induced phosphorylation of the receptors through GRKs (G protein-coupled receptor kinases) and the subsequent sequestration of the receptors from the cell surface (Krupnick et al., 1998) are not only important mechanisms for decreasing the signaling capacity of the receptor, but also play a key role in switching the receptor from G protein-dependent signaling pathway to G protein-independent signaling cascades (see Fig 5).

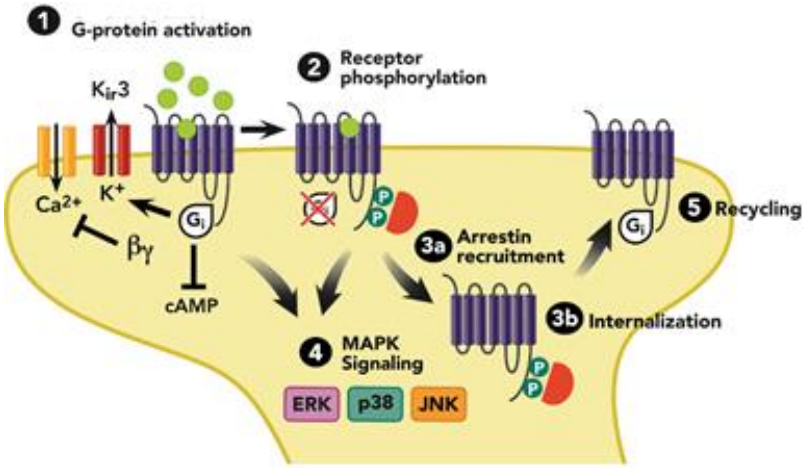

Figure 5. Signaling of GPCRs. *Extracted from Al-Hasaní and Bruchas, 2011*

1.4. Proteins that interact with GPCRs

Receptor stimulation by the appropriate ligand triggers a conformational change in the receptor that mediates the activation of the intracellular transduction machinery. There are two main different mechanisms involved in signal transduction of GPCRs: activation of G proteins and activation of the β-arrestin pathway.

1.4.1. G proteins

Despite the size and diversity of the GPCR superfamily, these receptors interact with a relatively small number of G proteins to initiate intracellular signaling cascades. In humans, there are 21 Gα subunits encoded by 16 genes, 6 Gβ subunits (5 plus a splice variant) encoded by 5 genes, and 12 Gγ subunits (Downes & Gautam, 1999). Heterotrimers are typically divided into four main classes based on the primary sequence similarity of the Gα subunit: Gαs, Gαi/o, Gαq and Gα12 (Simon et al., 1991). The molecular weights of the G protein subunits are: 39-46 kDa for the α subunit, 37 kDa for the Gβ and 8 kDa for the Gγ. G proteins are identified by their Gα subunits. Based on the sequence and functional similarities, Gα proteins are grouped into four families: Gαs, Gαi, Gαq and Gα12. In the Gαs family there are two members: Gαs is

expressed in most types of cells and Gαolf is specially expressed in the olfactory sensory neurons. Gαi family is the largest and most diverse family, including Gαi1, Gαi2, Gαi3, Gαo, Gαt, Gαg and Gαz. Gαi proteins haven been detected in most types of cells. Gαo is highly expressed in neurons and has two splice variants: GαoA and GαoB. Gαt has two isoforms: Gαt1 is expressed in the rod cells in the eye, while Gαt2 is in the cone cells of the eye. Gαg is found in taste receptor cells. Gαz is expressed in neuronal tissues and in platelets. In humans, the Gαq family consists of Gαq, Gα11, Gα14, and Gα16. Gαq and Gα11 are ubiquitously expressed, while Gα14 and Gα15/16 expression is more restricted. In the Gα12 family, there are Gα12 and Gα13, which are expressed in most types of cells. There are 5 Gβ and 12 Gγ genes in the human and mouse genomes. Gβ1, Gβ2, Gβ3, and Gβ4 share high sequence similarities (between 80 to 90%), while Gβ5 is ~50% similar to other Gβ subunits. While Gβ5 is mainly found in the brain, other Gβ subunits are widely distributed. β5 has a splice variant containing an additional 42-amino acid sequence (β5L). Gγ subunits are more diverse and share sequence similarities ranging from 20% to 80% (Syrovatkina et al., 2016).

Table 1 summarizes the localization of each G protein subunit in the human body. In terms of the brain, Gαi1, Gαi2 and Gαi3 and specially Gαo are expressed. Gαs and Gαq are also expressed widely. For the Gs family, there is evidence of contrasting brain expression pattern between Gαs and Gαolf (Hervé, 2011) but, to our knowledge, no clear region-specific pattern of mRNA expression for Gαi/o protein subtypes in the brain has been reported. Detailed characterization of the expression patterns for Gαi/o protein subtypes would then be central to determine their role in GPCR activation.

Name	Gene	Expression
α-Subunits		
$G\alpha_s$ class		
$G\alpha_s$	*GNAS*	Ubiquitous
$G\alpha_{sXL}$	(*GNASXL*)	Neuroendocrine
$G\alpha_{olf}$	*GNAL*	Olfactory epithelium, brain
$G\alpha_{i/o}$ class		
$G\alpha_{i1}$	*GNAI1*	Widely distributed
$G\alpha_{i2}$	*GNAI2*	Ubiquitous
$G\alpha_{i3}$	*GNAI3*	Widely distributed
$G\alpha_o$	*GNAO*	Neuronal, neuroendocrine
$G\alpha_z$	*GNAZ*	Neuronal, platelets
$G\alpha_{gust}$	*GNAT3*	Taste cells, brush cells
$G\alpha_{t\text{-}r}$	*GNAT1*	Retinal rods, taste cells
$G\alpha_{t\text{-}c}$	*GNAT2*	Retinal cones
$G\alpha_{q/11}$ class		
$G\alpha_q$	*GNAQ*	Ubiquitous
$G\alpha_{11}$	*GNA11*	Almost ubiquitous
$G\alpha_{14}$	*GNA14*	Kidney, lung, spleen
$G\alpha_{15/16}$	*GNA16* (*Gna15*)	Hematopoietic cells
$G\alpha_{12/13}$ class		
$G\alpha_{12}$	*GNA12*	Ubiquitous
$G\alpha_{13}$	*GNA13*	Ubiquitous
β-Subunits		
β_1	*GNB1*	Widely, retinal rods
β_2	*GNB2*	Widely distributed
β_3	*GNB3*	Widely, retinal cones
β_4	*GNB4*	Widely distributed
β_5	*GNB5*	Mainly brain
γ-Subunits		
γ_1, γ_{rod}	*GNGT1*	Retinal rods, brain,
γ_{14}, γ_{cone}	*GNGT2*	Retinal cones, brain
γ_2, γ_6	*GNG2*	Widely
γ_3	*GNG3*	Brain, blood
γ_4	*GNG4*	Brain and other tissues
γ_5	*GNG5*	Widely
γ_7	*GNG7*	Widely
γ_8, γ_9	*GNG8*	Olfactory/vomeronasal epithelium
γ_{10}	*GNG10*	Widely
γ_{11}	*GNG11*	Widely
γ_{12}	*GNG12*	Widely
γ_{13}	*GNG13*	Brain, taste buds

Table 1. G protein subunits. The name of the gene and the expression of each G protein subunit is indicated. *Extracted and modified from Wettschureck and Offermans, 2005.*

In terms of function, Gs and Gi regulate adenylyl cyclase positively or negatively, respectively, to control cAMP production. Gq activates phospholipase C (PLC) and promotes cleavage of phosphatidylinositol biphosphate (PIP2) into inositol triphosphate (IP3) and diacyl glycerol (DAG). IP3 mobilizes endoplasmic reticulum calcium via IP3 receptors (calcium channel) while DAG can activate protein kinase C (PKC). G12 activates RhoGTPase nucleotide exchange factors.

Many crystal structures of these G protein subunits have been resolved in various conformations, and provide the framework for understanding the biomechanics of G protein signaling (Sprang et al., 1997; Oldham and Hamm, 2006). The structures of the Gα subunit

reveal a conserved protein fold that is composed of a GTPase domain and a helical domain (Fig 6). The GTPase domain is conserved in all members of the G protein superfamily, and it hydrolyses GTP and provides the binding surfaces for the Gβγ dimer, GPCRs and effector proteins. In addition, this domain contains three flexible loops where significant structural differences between the GDP-bound and the GTPγS-bound (Noel et al., 1993, Coleman et al., 1994) conformations of Gα have been identified (Fig 6). The helical domain is unique to Gα proteins and is composed of a six α-helix bundle that forms a lid over the nucleotide-binding pocket, burying bound nucleotides in the core of the protein. All Gα subunits except Gαt, are post-translationally modified with the fatty acid palmitate at the N terminus. Members of the Gαi family are also myristoylated at the N terminus. These modifications regulate membrane localization and protein-protein interactions (Chen and Manning, 2001; Smotrys et al., 2004).

The Gβ subunit has a seven bladed β propeller structure. The N terminus of Gβ adopts and α-helical conformation that forms a coiled-coil with the N terminus of Gγ, and the C terminus of Gγ binds to blades five and six (Wall et al., 1995; Sondek et al., 1996) (Fig 6). All of the Gγ subunits undergo post-translational isoprenylation of their C termini with either a farnesyl or geranylgeranyl moiety. The G protein β- and γ-subunit form a functional unit that can only be dissociated under denaturing conditions (Schmidt et al., 1992). Although most Gβ subunits can interact with most Gγ subunits, not all the possible dimer combinations occur (Clapham et al., 1997). Additionally, several Gβγ dimers can interact with the same Gα isoform, which suggests that differential expression or subcellular localization are important in the regulation of downstream signaling (Graf et al., 1992).

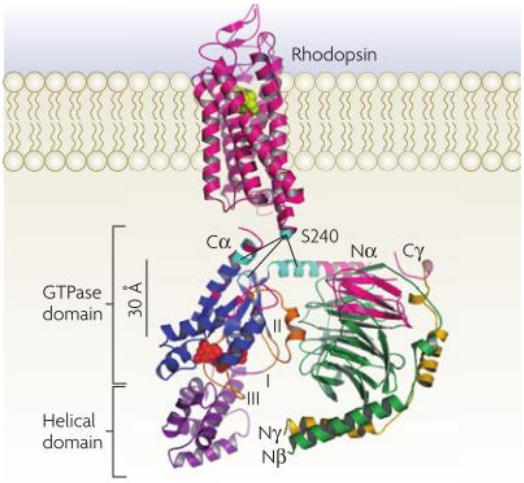

Figure 6. Receptor-G protein interface. Ribbon model of rhodopsin (Protein Data Bank (PDB) ID 1GZM) juxtaposed with the G_t heterotrimer (PDB ID 1GOT). Receptor activation exposes the G protein binding site that is formed by the intracellular loops of the receptor. Biochemical studies have identified several receptor contact sites on the G protein (pink and cyan). A few studies have also defined specific point-to point interactions, such as between Ser240 on intracellular loop 3 of the receptor (cyan sphere) with specific regions of the GαN terminus (Nα), C terminus (Cα) and α4–β6 loop (cyan). These contact regions, with the fatty acid modifications on the GαN terminus and GγC terminus, suggest a probable orientation with respect to the membrane. This orientation places GDP (red spheres) in the nucleotide-binding pocket on Gα(blue) ~30 Å from the sites of nearest receptor contact, posing the question of how receptors cause GDP release from this distance. *Extracted from Oldham and Hamm, 2008.*

It was long-believed that a given GPCR interacts with a particular G protein or a given family of G proteins. However, accumulating evidence has now clearly indicated that several GPCRs can simultaneously interact with G proteins that belong to different families and can activate different signaling cascades, some of which exert opposing effects. The efficacy of coupling to the various G proteins may then vary according to the receptor type and the interacting G protein but also depends on the agonist. Indeed, the observation that different agonists can affect which G proteins are activated by a given receptor supports a model where specific receptor conformations may be more or less favorable for coupling to specific G proteins (reviewed in Kenakin, 2003; Perez and Karnik, 2005).

Molecular mechanisms of G protein coupling to the receptor and subsequent activation of the heterotrimeric G protein are not fully understood despite the generally accepted G protein activation cycle described previously (see Fig 4). Two opposing models have been presented to explain how G proteins encounter activated receptors. In the "collision coupling" model, these interactions occur as a result of free lateral diffusion within the plasma membrane, wherein G proteins only interact with activated receptor (Tolkovsky and Levitzki, 1978). The alternative model suggests that G proteins can interact with receptors before agonist binding (that is, they are "precoupled"). The precoupling model is attractive because it can account for some of the specificity of receptor-G protein coupling and the rapid intracellular response that is observed. This model goes in line with the abundant experimental evidence indicating that the lateral motion of membrane components is very much constrained by various mechanisms that include interactions with the cytoskeleton and membrane microdomains, such as "lipid rafts". These are small microdomains (25-100 nm) that have decreased fluidity compared to other portions of the plasma membrane as a result of their high cholesterol and sphingolipid content (Pike, 2009). In fact, many GPCRs and their cognate signaling proteins (most G protein subunits and AC isoforms) have been localized to the lipid rafts (Cooper and Crossthwaite, 2006; Allen et al., 2007; Patel et al., 2008). Enrichment and compartmentalization of GPCR signaling components in lipid rafts could then be a universal mechanism for increasing the effective concentration of these proteins by restricting their movement and favoring their specific intermolecular interactions and, consequently, the transmission of the sequence of conformational changes initiated by the agonist and leading to the enzymatic response (Patel et al., 2008).

Another important aspect is the conformational status of the activated Gα subunit. Although subunit dissociation in the heterotrimer (Gα and βϒ dissociation) has classically been accepted as the necessary step for functional coupling to the AC due to the overlap of binding interface between Gα-βϒ and Gα-AC (Oldham and Hamm, 2008), accumulating evidence suggests that the G protein heterotrimer may not dissociate upon activation of the receptor and the associated heterotrimer can still signal (Galés et al., 2006; Hein and Bunemann, 2009; Lohse et al., 2012; Ferré, 2014,2015). Much of the recent data in favor of the precoupling hypothesis comes from FRET and BRET studies using labelled receptors and G protein subunits to study these interactions *in vivo* and *in vitro*. Experiments done with G protein subunits (α and γ)

fused to RET donor and acceptor molecules indicate that G protein activation upon ligand binding to the receptor does not lead to dissociation, but implies a conformational change with rearrangement and reorientation of its subunits (Galés et al., 2006; Hein et al., 2006). The structural model of G protein activation proposed by Michel Bouvier's research group (Galés et al., 2006) implies a significant modification of the quaternary structure of the heterotrimeric G protein; a reorientation without dissociation of the subunits that is still compatible with the existence of overlapping binding sites of Gα for AC and Gβγ. Their findings suggest that during G protein activation Gβγ is maintained in the heterotrimeric complex while being displaced away from its original site of association with Gα (Galés et al., 2006 and Fig 7).

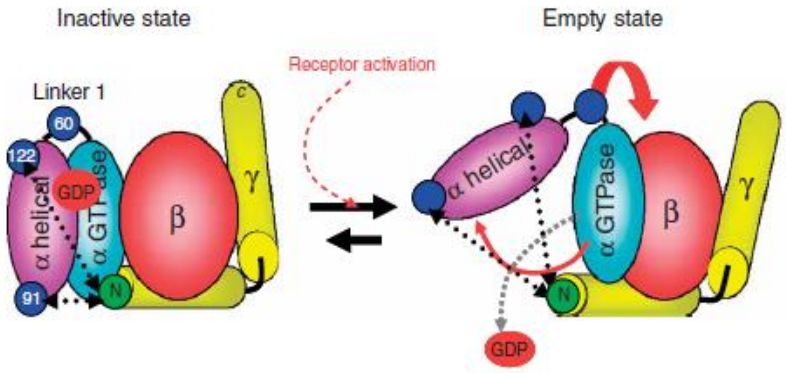

Figure 7. Schematic representation of structural rearrangement within Gαi1-β1-Y2 detected by BRET after receptor activation. Scheme depicts opening of the Gαi1 GTPase and helical domain through linker 1 (like a clamp). This and other rearrangement within the G protein would thus create an exit route for the GDP. *Extracted from Galés et al., 2006.*

1.4.2. Arrestins

Following prolonged ligand stimulation, GPCRs are desensitized to attenuate continued signaling. This process involves blockade of further G protein activation and subsequent receptor internalization, both of which are mediated by arrestin adaptor proteins.

First, members of a protein family known as the G protein-coupled receptor kinases (GRKs) rapidly phosphorylate the receptor, typically on its cytoplasmic tail. Phosphorylated receptors

present high-affinity binding surfaces to recruit the cytosolic adaptors, β-Arrestins. Steric binding by β-Arrestin interferes with further G protein coupling leading to desensitization of G protein-dependent signaling. β-Arrestins also scaffold the second messenger degrading enzymes such as phosphodiesterase 4D, PDE4D, that degrades cAMP and diacylglycerol kinase, DAG-K, that converts diacylglycerol to phosphatidic acid. In parallel, agonist stimulation promotes rapid internalization of cell-surface GPCRs into clathrin-coated vesicles. This internalization is facilitated by β-arrestin binding, which has specific binding domains for clathrin and AP2. β-arrestin binding to other endocytic proteins (NSF and Arf6) is also required for efficient receptor internalization. The interaction between β-arrestin and the E3 ubiquitin ligase Mdm2 promotes ubiquitinization of β-arrestin, which facilitates robust binding of β-arrestin with both cargo (GPCR) as well as endocytic machinery (clathrin and AP2). Receptor internalization is followed by postendocytic sorting of internalized receptors for recycling or lysosomal degradation. The third aspect mediated by β-arrestin is signaling. β-arrestin acquires an active conformation upon forming a complex with agonist-stimulated 7TMRs and scaffolds proteins of the MAPK family, a family of serine/threonine kinases that include ERK1/2 (also known as p44/p42 MAPK), p38 kinases and the c-Jun N-terminal kinases. The downstream effectors of MAPKs control many cellular functions including cell cycle, regulation of transcription and apoptosis. The multiple functions of β-arrestins are summarized in Fig 8.

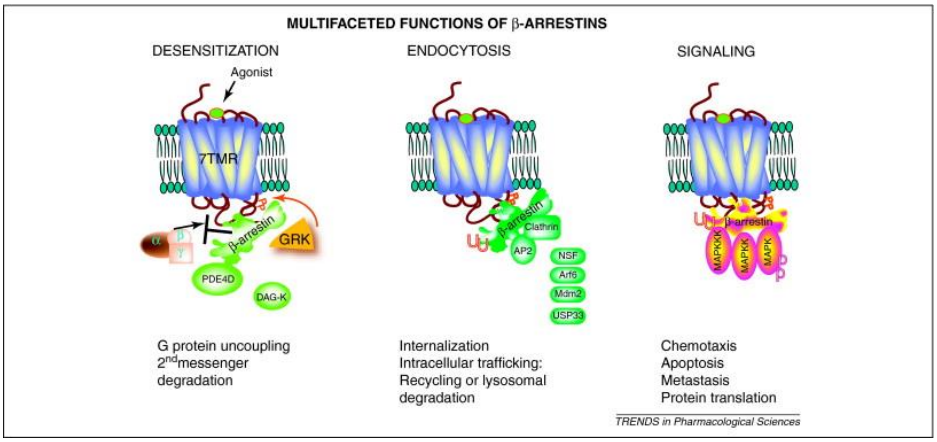

Figure 8. Multifaceted functions of β-arrestins. *Extracted from Shenoy and Lefkowitz, 2011.*

There are four subtypes of arrestin: Arrestin-1 and Arrestin-4 are restricted to the retina, whereas arrestin-2 (β-Arrestin 1) and Arrestin-3 (β-Arrestin 2) are ubiquitous expressed in all cells and tissues and function in the desensitization of most GPCRs.

In humans, seven GRKs are classified into three subfamilies. The GRK1-like family includes GRK1 and GRK7, which primarily regulate photoreceptors in the retina. The GRK2-like family includes GRK2 and GRK3, both of which are ubiquitously expressed. The GRK4-like subfamily includes GRK4, GRK5 and GRK6.

1.5. Functional selectivity

GPCRs do not act as simple switches that turn a single signaling pathway "on" or "off". Instead, individual receptors engage multiple signaling cascades and individual ligands can have differential efficacies toward subsets of these signaling effectors. For instance, one ligand can be a full agonist for the activation of the G protein pathway and an antagonist or partial agonist for activation of the β-arrestin pathway. This concept contradicts the classical idea in which the agonist has the capacity to activate equally all signaling pathways. However, thanks to new pharmacological tools it has been demonstrated that many agonists do not activate receptors through stabilization of the same active state, but they stabilize unique active states to create a signal that is "biased" towards specific cellular pathways. This phenomenon, known as ligand-biased signaling or functional selectivity, is revolutionizing seven-transmembrane receptor drug discovery since it offers interesting opportunities to identify and develop compounds with increased selectivity and improved safety profiles (Kenakin, 2011; Urban et al., 2007; Kenakin and Christopoulos, 2013).

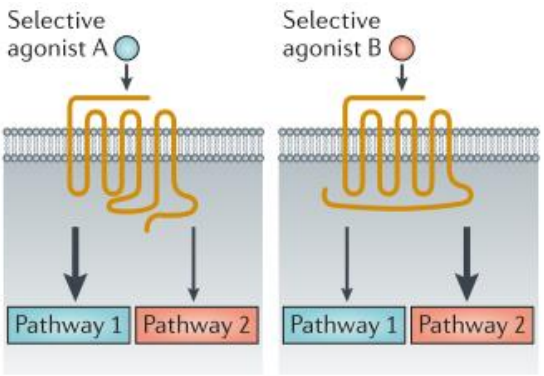

Figure 9. Biased agonism or functional selectivity: agonist A produces a biased stimulus for cellular signaling pathway 1, whereas agonist B stabilizes another receptor conformation that selectively induces bias for cellular signaling pathway. *Extracted from Kenakin and Christopoulos, 2013.*

An early example of biased agonism was demonstrated with two agonists of the muscarinic acetylcholine receptor: carbachol and pilocarpine. Binding of carbachol results in a balanced response that is mediated by both Gαs and Gαq. By contrast, other ligands such as pilocarpine do not lead to Gs-mediated adenylyl cyclase stimulation, but do lead to phospholipase C activity that is mediated by Gq (Fisher et al., 1993, Gurwitz, 1994). The ability of one receptor to couple to multiple G proteins and activate multiple signaling pathways has also been demonstrated for α_2-adrenergic receptor subtypes which have been shown to couple to both Gαs and Gαi (Eason et al., 1992, 1995). Moreover, signaling through these parallel pathways can differ depending on the ligand used to stimulate the receptor, thereby resulting in a biased response (reviewed in Roth, 2009). Although this concept is under development, it opens an exciting new field when thinking about compounds that can target specific receptor-G protein subtype complexes. This is important when considering that specific G proteins subtypes might be expressed differentially depending on the cell population. This concept will be further explored in the first and second chapter of the Results section of this thesis.

Several groups have observed functional selectivity in the serotoninergic system. The effector that has been best characterized with respect to 5-HT_2 signaling involves the Gq stimulation of phospholipase C (PLC), leading to the formation of inositol phosphates (IP) and diacylglycerol (DAG), thereby modulating intracellular calcium (Hoyer et al., 1994). This receptor family also has been shown to mediate the release of arachidonic acid (AA)

presumably through the activation of phospholipase A_2 (PLA_2) (Felder et al., 1990). Berg et al., (1998) reported the capability of certain serotoninergic ligands to differentially activate these signaling pathways associated with 5-HT_{2A} and 5-HT_{2C} receptors. They observed that the relative efficacy of a series of ligands for each of the receptors differed depending upon whether PLC-mediated accumulation of IP or PLA_2-mediated release of AA was measured. For example, bufotenin acts as full agonists at AA release and a partial agonist at IP accumulation whereas TFMPP is a full agonist at IP accumulation but has only partial intrinsic efficacy at AA release. Another example from studies looking specifically at hallucinogens and their differential signaling through the 5-HT_{2A} receptor compared the effects of LSD and DOB (a hallucinogenic phenetylamine derivative) on 5-HT_{2A} receptor-mediated accumulation of IP_3 and found that both had significant potency for this pathway, yet LSD showed little intrinsic efficacy relative to that of DOB (Kurrasch-Orbaugh et al., 2003).

Dihydrexidine (DHX) and its congener N-propylDHX were initially characterized as full agonists at the D_2 receptor, because they were as efficacious as dopamine in inhibiting cAMP synthesis and efflux in striatal slices, inhibiting prolactin release *in vivo*, and stimulating GTPγS binding in rat substantia nigra (Mottola et al., 1992; Kilts et al., 2002). However, further characterization of the functional profile of these compounds demonstrated that they are not typical full D_2 receptor agonists. Neither DHX nor N-propylDHX is able to inhibit the synthesis and release of dopamine in rat striatum or inhibit the firing of nigral dopaminergic neurons, effects that would be expected for typical D_2 receptor agonists (Mottola et al., 1992; Kilts et al., 2002).

Although opioids are useful analgesics, they can also produce respiration depression, an effect that is linked to the activation of β-arrestin (Raehal et al., 2005). Therefore, an opioid agonist that stimulates opioid analgesia pathways without promoting receptor-β-arrestin interactions would be predicted to have fewer side effects (Bohn et al., 1999; Xu et al., 2007; Groer et al., 2007). As a matter of fact, in a recent study published in Nature (Manglik et al., 2016), a biased agonist called PZM21 was shown to be a potent Gi activator with exceptional selectivity for μ-opioid receptor and minimal β-arrestin 2 recruitment. Unlike morphine, PZM21 is efficacious for the analgesia and is devoid of both respiratory depression and morphine-like reinforcing activity in mice (Manglick et al., 2016).

At present, the most advanced functionally selective molecule is the biased angiotensin ligand TRV120027 (Boerrigter et al., 2011, 2012). *In vivo* treatment with this molecule blocks angiotensin, which reduces blood pressure, but also promotes the beneficial cardiac effects of β-arrestin activation via stimulation of p42 and/or p44 MAPK, SRC and endothelial nitric oxide synthase phosphorylation and is thus predicted to have a therapeutic advantage compared to classical angiotensin antagonists (Rajagopal et al., 2006; Wei, 2003; Aplin et al., 2009; Violin and Lefkowitz, 2007; Zhai, 2005; Boerrigter et al., 2011, 2012).

Although functionally selective drugs such as this and others open a new and exciting field, it is not yet clear to what extent this effect can be exploited for therapeutic advantage. Ultimately, as more functionally selective molecules enter the clinic, translational medicine will enable association of pharmacological functional selectivity with therapeutic advantage.

2. GPCR oligomerization

2.1. Evidence for GPCR oligomerization

Although G protein-coupled receptors were initially thought to be monomeric entities, evidence accumulated over the past three decades indicates that they can form homomers and heteromers in intact cells (Bouvier, 2001; Milligan and Bouvier, 2005; Pin et al., 2007; Ferré et al., 2009; Guitart et al., 2014; Ferré, 2014, 2015; Bonaventura et al., 2015; Navarro et al., 2015). A receptor heteromer is a macromolecular complex composed of at least two functional receptor units with biochemical properties that are demonstrably different from those of its individual receptors. It is now well accepted that family C GPCRs (e.g., metabotropic glutamate, calcium-sensing receptors, $GABA_b$ and sweet and umami taste receptors) form constitutive homo- or heteromers (Kniazeff et al., 2011). Such observations raised a long debated question about whether family A (rhodopsine-like) GPCR dimers were also constitutive and necessary for G protein activation. An explosion of data supporting the existence of homo- and heteromers of GPCRs in intact cells came with the widespread use of biophysical techniques such as resonance energy transfer, fluorescence complementation or combination of these techniques (Milligan and Bouvier, 2005; Gandía et al., 2008; Pin et al., 2007, 2009; Bacart et al., 2008; Carriba et al., 2008; Guo et al., 2008; Cabello et al., 2009; Ferré et al., 2009; Urizar et al., 2011). These techniques however, have largely fallen short in answering questions about the size of the oligomer complexes and their possible dynamic nature. Initial evidence for GPCR oligomerization in a physiologically relevant system came from atomic force microscopy experiments in native disk membranes from mice, which showed rhodopsin to be arranged in paracrystalline array of dimers (Fotiadis et al., 2003). These studies raised severe criticism (Chabre and le Maire, 2005), and more recent studies using single molecule techniques have begun to address the details of the spatial and temporal organization of GPCR complexes in living cells by directly observing the state and behavior of individual proteins in the cell. Single-molecule total internal reflectance fluorescence microscopy (TIRFM) was first used to track the position of individual molecules of muscarinic acetylcholine M_1 and N-formyl peptide receptors using fluorescently labeled ligands (Hern et al., 2010; Kasai et al., 2011). Both studies suggested a transient (second-scale)

formation and dissociation of dimers, with 30-40% proportion of dimers at any given time. Despite some concerns, similar studies have reached equivalent conclusions on the state of the muscarinic acethylcholine M_2 receptor in cardiac muscle (Nenasheva et al., 2012). More recently, TIRFM was used together with SNAP-tag technology to directly label cell-surface GPCRs with organic fluorophores to dynamically monitor individual β_1- and β_2-adrenoceptors as well as $GABA_b$ receptors on the surface of living, transiently transfected cells (Calebiro et al., 2013). This study shows that all three receptors form dimers and higher-order oligomers (also with estimations of dynamic second-scale receptor-receptor interactions) in a proportion dependent on the subtype of receptor and on the receptor density. At low densities, monomeric species were predominant for the β_1-adrenoceptor, whereas β_2-adrenoceptors displayed a higher proportion of dimers. Importantly, at densities comparable to receptor expression in native tissue, dimers and higher-order oligomers were predominant species for both adrenergic receptors (Calebiro et al., 2013). Fluorescence correlation spectroscopy (FCS) is another indirect but useful technique to determine the oligomer status of protein clusters (Chen et al., 2003). It has been successfully used to study μ-opioid receptor homo- and heteromerization with δ-opioid receptors and the data suggested that μ-opioid receptors exist primarily as dimers that oligomerize with δ-opioid receptors into tetramers (Golebiewska et al., 2011). The recent FCS with a particle counting histogram approach by Herrick-Davis et al. (2013) also provides support for homodimers being the predominant, and perhaps only, species for several GPCRs, including α_{1B}-adrenoceptor, β_2-adrenoceptor, serotonin 5-HT_{2A} and 5-HT_{2C}, muscarinic acetylcholine M_1 and M_2, and dopamine D_1 receptors. Stability of GPCR dimers/oligomers is inferred from experiments that indicate these complexes are generated at an early stage of biosynthesis. In fact, it has been suggested that early-stage dimerization may be required for effective folding and maturation of the receptor (Salahpour et al., 2004; Bulenger et al., 2005; Milligan, 2010,2013). The same reasoning has been used based on evidence for cointernalization, with the capacity of a selective ligand of one of the receptors in a GPCR heteromer to cointernalize the two different receptors constituting the heteromer (Hillion et al., 2002; Milligan, 2010, 2013; Ward et al., 2011; Tadagaki et al., 2012).

Negative results should also be acknowledged, such as the recent study by Gavalas et al. (2013) which showed evidence for interaction between metabotropic glutamate receptors

but not β_2-adrenergic or μ-opioid receptor. Apart from differences between receptors or when considering receptor homomers or heteromers, it might be that a number of the approaches used to study oligomerization are unable to resolve fluctuations in receptors interactions occurring on a second or subsecond scale. On the other hand, mammalian transfected cells might be lacking elements that increase the stability of the interfaces that determine the receptor-receptor interactions, which might also be different depending on the cellular compartment studied (Ferré et al., 2014).

Distinct intermolecular interactions have been found to be involved in various GPCRs homo- and heteromers. Recently calculated estimates of the relative stability of different dimeric interfaces of different GPCR subtypes using extended biased molecular dynamics simulations in explicit lipid-water environments argue in favor of a variable strength of association depending on the specific residue composition or shape of the interface, despite an overall transiency in receptor-receptor interactions (Johnston et al., 2012). Notably, simulations of β_1- and β_2-adrenoceptors suggested a model of oligomerization in which more stable homodimers involving TM1 diffuse through the membrane and transiently interact with other protomers/dimers involving other TM helices. In agreement with these predictions are the results obtained in a recent study with the muscarinic acetylcholine M_3 receptor, using quantitative FRET spectrometry techniques with controlled expression of the energy donor-tagged species (Patowary et al., 2013). Mathematical analysis of the FRET efficiencies obtained from spectral unmixing was compatible with the M_3 receptor existing as stable dimeric complexes, a large fraction of which interacted dynamically to form tetramers that were specifically within a rhombic organization rather than a square or linear configuration (Patowary et al., 2013).

A very intriguing set of inferences in the field of GPCR oligomerization has been based on recently obtained high-resolution crystallographic structures, including those of the chemokine CXCR4, the μ-opioid and κ-opioid receptors, the β_1-adrenoceptor and the smoothened receptor (Wu et al., 2010, 2012; Manglik et al., 2012; Huang et al., 2013; Wang et al., 2013). Each of these crystallized as parallel dimers and/or tetramers. When looking at the interfaces between this protomers, TM5 and TM6 constituted the main interfaces for chemokine CXCR4 and μ-opioid receptor crystallized dimers (Wu et al., 2010; Manglik et al., 2012). Apart from the TM5-TM6 interface, crystallized chemokine CXCR4 dimers also showed

contacts at the intracellular ends of TM3 and TM4 (Wu et al., 2010) and μ also showed another interface formed by TM1, TM2 and H8 (Manglik et al., 2012). Involvement of TM6 was also suggested early on for β_2-adrenoceptor dimers (Hebert et al., 1996) and for the leukotriene receptor BLT1 (Baneres and Parello, 2003) by the use of interfering synthetic peptides with the same sequence as TM6. Another study suggested that TM4 was the main interface in the D_2 oligomer (Guo et al., 2005). Another study found two interfaces in serotonin 5-HT_{2C} receptor dimers involving TM4-TM5 and TM1. In summary, although a pattern of similar interfaces of GPCR homomers seems to be emerging, different interfaces can be found in different oligomers and even in different conformations of the same oligomers. Another important aspect is whether agonist can modify GPCR oligomeric interfaces and therefore the dynamics of receptor oligomerization.

RET techniques are good methods to study the architecture of heteromers in transfected living cells. Bimolecular fluorescence complementation (BiFC) combined with application of TM peptides with the amino acid sequence of receptors was used to determine the interfaces of different receptor heteromers. In the study by Guitart et al. (2014) application of TM5 and TM6 corresponding to D_1 receptor was shown to modify the quaternary structure of the D_1-D_3 heteromer (Guitart et al., 2014). Later on, Bonaventura et al. (2015) shown that application of TM5, but not TM7, of A_{2a} or D_2 was able to destabilize receptor heteromerization, as suggested previously (Borroto-Escuela et al., 2010). Another example was described with the corticotropin-releasing factor (CRF_1) and orexin (OX_1) receptor heteromer where TM5 and TM1 were able to disrupt the interaction between these two receptors (Navarro et al., 2015). Finally, TM5 and TM6 of CB_1 were found to be involved in the 5-HT_2A-CB_1 interface (Viñals et al., 2015). In all cases the TM7 of the corresponding receptors did not affect the BiFC of the heteromers.

A part from interactions between TM domains, several studies have provided evidence for disulfide bridges between extracellular domains of class C GPCR homomers (Kniazeff et al., 2011) and for a key role of electrostatic interactions between intracellular receptor domains in receptor heteromerization (Woods and Ferré, 2005). These electrostatic interactions have been suggested to be involved in several receptor heteromers (Ciruela et al., 2004; Woods and Ferré, 2005; Navarro et al., 2010; O'Dowd, 2012, 2013). An example of a functionally relevant electrostatic interaction is the one involved in adenosine A_{2a}-dopamine D_2 receptor

heteromer, with an arginine-rich domain localized in the N-terminal portion of the long third intracellular loop of the dopamine D_2 receptor and the acidic domain in the distal part of the long C-terminus of the adenosine A_{2a} receptor (Ciruela et al., 2004; Navarro et al., 2010). Mutation- or peptide-mediated disruption of the A_{2a}-D_2 receptor electrostatic interaction produces a profound destabilization of the quaternary structure of the heteromer (Navarro et al., 2010) with disappearance of significantly relevant A_{2a}-D_2 receptor interactions in brain tissue, such as the adenosine A_{2a} receptor-mediated inhibition of dopamine D_2 receptor-induced depression of striatal neuronal firing (Azdad et al., 2009).

In summary, most evidence indicates that, as for family C GPCRs, which can be found as strict homodimers, heterodimers (Doumazane et al., 2011), and as stable heteromers (Comps-Agrar et al., 2012), family A GPCRs form homo- and heteromers in heterologous systems. Homodimers seem to be a predominant species with potential dynamic formation of higher-order oligomers, particularly tetramers. Is still needs to be resolved if class A GPCR heteromers are preferentially heterodimers or if they are mostly constituted by heteromers of homodimers.

2.2. Pharmacological and functional aspects of GPCR oligomerization

It has long been recognized that agonists show “binding heterogeneity”: a wide diversity of affinities for GPCRs in membrane preparations from transfected mammalian cell lines or from native tissues. The typical example is a competition experiment between a moderate concentration of a radiolabeled antagonist and increasing concentrations of the agonist, which results in a non-steep or even biphasic curve, apparently indicating the existence of two populations of GPCRs with high and low affinities for the agonist.

In line with the classical view of GPCR signal transduction, a still common view of GPCR signaling holds that agonists promote the formation of a transient complex between the monomeric receptor and the G protein, with conformational changes in both molecules that lead to an increased affinity of the receptor for the agonist and for the heterotrimeric G protein, which lead to G protein activation. The classical model of ligand-GPCR binding (De Lean et al., 1980) proposes that the receptor-G protein complex accounts for the high-affinity

site for the agonist, while the G protein-uncoupled receptor accounts for the low-affinity site. Guanylyl nucleotides would therefore, promote uncoupling of the receptor-G protein complex, converting all receptors into low-affinity sites. According to the model, a non-steep or biphasic antagonist-agonist competitive inhibition curve implies the existence of two populations of receptors: coupled and non-coupled to the G protein. Implicitly, this assumes a limited pool of G proteins, an assumption difficult to reconcile with the fact that the expression levels of G proteins in native cell systems exceeds those of GPCRs (Neubig, 1994). An alternative model that could explain complex radioligand curves is the "two-state dimer model" that considers GPCR oligomerization, or at least GPCR dimers (Casadó et al., 2007; Ferré et al., 2014). Allosteric communication through the two GPCR units or protomers allows negative or positive cooperativity, meaning that the binding of a ligand to the first protomer decreases or increases the affinity of the ligand for the second protomer. This mechanism does not depend on a limited pool of G proteins, which can always be coupled and act as additional allosteric modulators to increase the affinity of the agonist, thereby generating a dimer conformation that allows ligand cooperativity through the protomers (Ferré et al., 2014, 2015).

The "two-state dimer model" (Casadó et al., 2007, 2009) provides a better approach than the monomer-G protein models for fitting data that eventually give more accurate and physiologic relevant parameters. In terms of accuracy, the two-state dimer model is significantly more robust than the two-independent site model. Thus, with the two-state dimer model the same parameters (RT, equilibrium dissociation constants) are obtained irrespective of the concentration of radioligand (Casadó et al., 2009). In terms of physiological relevance, the two-state dimer model does not only give equilibrium dissociation constants for high- and low-affinity binding to receptor dimers, but provides indexes of ligand cooperativity and of allosteric modulation between different ligands simultaneously binding to the dimer. Nevertheless, when looking for the most appropriate model, the best approach would be implementing both a monomer-based model and a two-state dimer model and comparing results. It is important to realized that choosing either model should not imply discarding the possibility of the existence of mixtures of monomers and dimers/oligomers.

In addition to ligand-binding properties, unique allosteric properties for each GPCR homodimer emerge in relation to intrinsic efficacy (the power of the agonist to induce a

functional response). Most experimental data agree with the model that proposed that ligand occupancy to the first protomer is enough to produce significant G protein activation and a functional response. When a ligand binds to the second protomer in the homodimer, it will often act as an allosteric modulator of the intrinsic efficacy of the ligand bound to the first protomer, by potentiating or reducing the functional response (Ferré et al., 2014, 2015). Furthermore, in addition to considering the allosteric modulations of an orthosteric agonist (ligand binding to the same site in the receptor where the endogenous ligand binds) binding to the first protomer or the same ligand binding to the orthosteric site in the second protomer, a significant number of possible pharmacological allosteric modulations of the homodimer appear when considering other ligands such as positive and negative allosteric modulators (ligand that binds to a different site in the receptor) and even bitopic ligands (ligands that occupy the allosteric and orthosteric sites at the same time) (Lane et al., 2014).

Heteromerization opens a new dimension of possible molecular and functional protein interactions within a complex. For example, the extensively studied adenosine A_{2a}-dopamine D_2 receptor heteromer provides a framework for the realization of the scope of these interactions. When considering GPCR heteromers as conduit of allosteric interactions, two possible scenarios should be considered (Kenakin and Miller, 2010). In the first scenario, binding of a ligand to one of the protomers leads to changes in the properties (affinity or intrinsic efficacy) of a ligand binding to the second protomer. A good example is provided by the allosteric antagonistic interaction between A_{2a} receptor agonists and antagonists on D_2 receptor agonists in the A_{2a}-D_2 receptor heteromer, wherein A_{2a} receptor agonists decrease the affinity and intrinsic efficacy of D_2 agonists (Ferré et al., 1991; Azdad et al., 2009; Bonaventura et al., 2015). The A_{2a}-D_2 receptor heteromer is selectively localized in the GABAergic striato-pallidal neurons (indirect pathway), where it plays a seminal role controlling basal ganglia function (Azdad et al., 2009; Trifilieff et al., 2011; Ferré et al., 2011). It has been hypothesized that these allosteric interactions between A_{2a} and D_2 receptor agonists within the A_{2a}-D_2 heteromer provide a mechanism to understand the behavioral depressant effects of adenosine analogs as well as the psychostimulant effects of selective A_{2a} receptor antagonist and the non-selective adenosine receptor antagonist caffeine, with implications for several neuropsychiatric disorders (Ferré et al., 2011). In fact, the same

mechanism provided the main rationale for the use of A_{2a} receptor antagonists such as KW 6002, in Parkinson's disease (Armentero et al., 2011; Jorg et al., 2014).

The second scenario of allosteric modulations within GPCR heteromers suggests a ligand-independent modulation in which one of the protomer acts as a modulator of ligand binding to the other molecularly different protomer (Kenakin and Miller, 2010). The A_{2a}-D_2 receptor heteromer again provides a valuable example. Screening with various *in vitro* and *in vivo* techniques led to the finding of very different qualitative properties of several selective A_{2a} receptor antagonists. The most striking finding was a selective decrease in the affinity of SCH 442416 when A_{2a} receptor forms heteromers with D_2 receptor, compared to when it does not heteromerize or when it forms heteromers with the adenosine A_1 receptor (Orrú et al., 2011). In competitive experiments with the orthosteric A_{2a} receptor antagonist [^{3}H]ZM 241385, binding of SCH 442416 demonstrated strong negative cooperativity when A_{2A} receptor was coexpressed with the D_2 receptor (Orrú et al., 2011). The implications of these results are several. First, not only agonists but also antagonists can manifest binding heterogeneity with negative cooperativity. Second, evidence for D_2 receptor-dependent allosteric modulation that determines negative cooperativity of a ligand binding to an A_{2a} receptor oligomer strongly suggest that the A_{2a}-D_2 receptor heteromer comprises at least two A_{2a} protomers (Ferré et al., 2014, 2015; Bonaventura et al., 2015).

The studies on A_{2a}-D_2 receptor heteromers can be better explained by a model that considers heteromers of homodimers coupled to their preferred G protein, a heterotetramer (Ferré et al., 2015). This structure provides the possibility of simultaneous binding of Gs and Gi proteins to their respective preferred receptors, generating an optimal framework for canonical Gs-Gi interaction. This could be a general scheme that should apply to other, if not all, GPCR heteromers containing both preferred Gs- and Gi-coupled receptors. The same scheme was recently reported for the dopamine D_1-D_3 receptor heteromer (Guitart et al., 2014). D_1 and D_3 receptors couple preferentially to Gs/olf and Gi proteins, respectively, and they are also localized in striatal cells (in the striato-nigral neuron or direct pathway). By combining RET techniques with complementation, it was demonstrated in the D_1-D_3 receptor heteromer that agonist binding to the D_1 or the D_3 receptor engages Gs or Gi proteins, respectively (Guitart et al., 2014). RET with double complementation of RET sensors was then used to uncover the tetrameric structure of the D_1-D_3 receptor heteromer. The same approach has been recently

used to identify A_{2a}-D_2 receptor heterotetramers (Bonaventura et al., 2015). By using peptides with the sequence complementary to specific TM domains of A_{2a} or D_2, the quaternary structure of the heteromer and its biochemical properties was disrupted (Bonaventura et al., 2015).

3. Dopamine receptors

3.1. Structure and synthesis of catecholamines

A catecholamine is a monoamine, an organic compound that has a catechol (benzene with two hydroxyl side groups at carbons 1 and 2) and a side-chain amine. Catecholamines are derived from the amino acid tyrosine, which is derived from dietary sources as well as synthesis of phenylalanine. Included among this group are epinephrine (adrenaline), norepinephrine (noradrenaline), and dopamine. Dopamine is incapable of crossing the blood-brain barrier so it must be synthesized inside the brain to perform its functions. Norepinephrine is synthesized and released by the central nervous system and also by a division of the autonomic nervous system called the sympathetic nervous system. Finally, epinephrine is normally produced by both the adrenal glands and certain neurons.

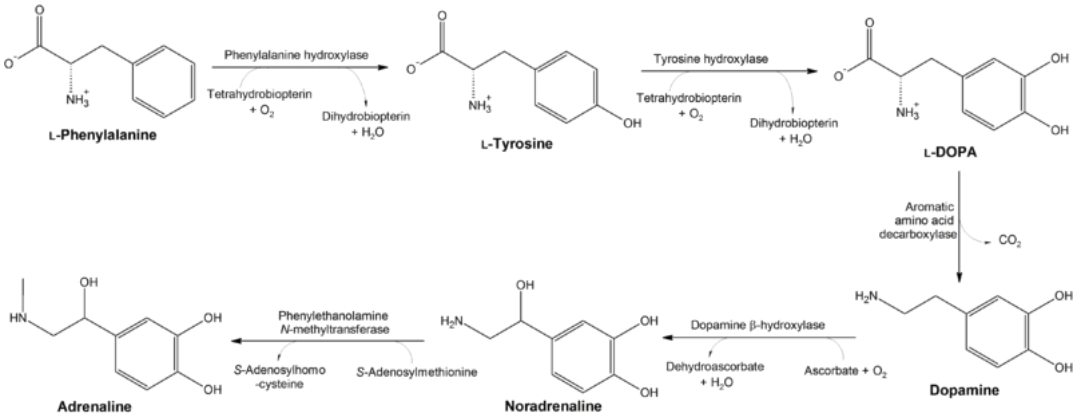

Figure 10. Synthesis of the catecholamines dopamine, norepinephrine and epinephrine.

As mentioned above, tyrosine can be either created from phenylalanine by hydroxylation mediated by the enzyme phenylalanine hydroxylase or it can also be ingested directly from dietary protein. Tyrosine is converted to DOPA by tyrosine hydroxylase followed by conversion of DOPA to dopamine by DOPA decarboxylase. Finally, dopamine is transported from the cytoplasm into vesicles by the vesicular transporter, VMAT2 (Eiden et al., 2004). Dopamine is stored in these vesicles until it is ejected into the synaptic cleft by voltage-dependent calcium channels that trigger the fusion of the dopamine filled vesicles with the

presynaptic membrane. Cells expressing dopamine β-hydroxylase (DβH) may convert dopamine into norepinephrine which are in turn stored in these vesicles until it is ejected into the synaptic cleft.

Once in the synapse, dopamine binds to and activates dopamine receptors. These can be postsynaptic dopamine receptors or presynaptic autoreceptors. Upon binding, the activation of the receptor triggers a complex chain of intracellular events that ultimately will lead to the activation or inhibition of the postsynaptic neuron. Finally, the dopaminergic signaling is terminated through the reuptake of dopamine from the synaptic cleft to the presynaptic terminal by the dopamine transporter (DAT). Once back in the cytosol, dopamine can either be broken down by the monoamine oxidase enzyme (MAO) or repackaged into vesicles by VMAT2 making it available for future release (Amara et al., 1993; Cooper et al., 1996; Eiden et al., 2004) (Fig 11).

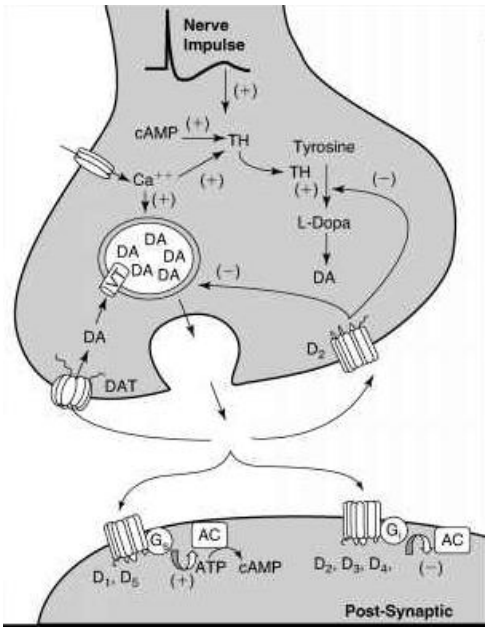

Figure 11. Schematic model of the mechanisms involved in dopamine synthesis, release, storage, reuptake and receptor activity in presynaptic and postsynaptic neurons. DA, dopamine; AC, adenylyl cyclase; TH, tyrosine hydroxylase; VT, vesicular monoamine transporter.

Norepinephrine also binds to and activates postsynaptic adrenergic receptors. To terminate its action, norepinephrine is absorbed back into the presynaptic neuron via reuptake mediated mainly by the norepinephrine transporter (NET). Once back in the cytosol,

norepinephrine can either be broken down by MAO or repackaged into vesicles by VMAT, making it available for future release. At the same time, α_2-adrenoceptors and probably β_2-adrenoceptors located in the presynaptic terminal behave as autoreceptors by inhibiting further norepinephrine release (Starke et al., 1989; Kanagy, 2005; Hein, 2006) (Fig 12).

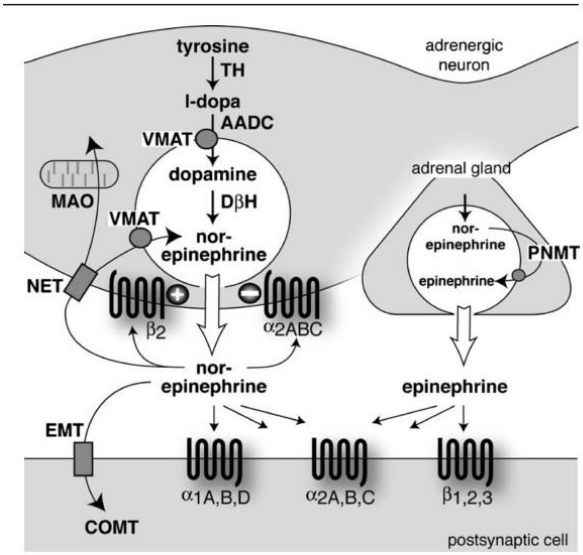

Figure 12. Steps involved in the synthesis and release of norepinephrine. While the conversion of tyrosine to dopamine occurs predominantly in the cytoplasm, the conversion of dopamine to norepinephrine by DβH occurs predominantly inside neurotransmitter vesicles. TH, tyrosine hydroxylase; AADC, aromatic amino acids decarboxylase; DβH, dopamine β-hydroxylase. *Extracted from Hein, 2006.*

3.2. Dopamine system in the brain

Since the discovery of the physiological functions of 3-hydroxytyramine (dopamine) almost 60 years ago (Carlsson et al., 1957), this catecholaminergic neurotransmitter has attracted an enormous amount of attention. Although dopaminergic neurons are rare (<1/100000 brain neurons), they regulate several important aspects of basic brain function including locomotor activity, motivation, memory and endocrine regulation. Dopamine also plays an important role in the brain reward system that controls and stimulates the learning of many behaviors (Girault and Greengard, 2004). In the periphery, this catecholamine also plays multiple roles as a modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility (reviewed in Missale et al., 1998: Sibley, 1999; Iversen and Iversen, 2007).

Brain areas that synthesize dopamine have projections that give rise to four axonal pathways, namely nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular systems (reviewed in Iversen and Iversen, 2007). The nigrostriatal pathway is formed by projections that arise from dopamine-synthesizing neurons of the midbrain nucleus, the substantia nigra compacta, which innervates the dorsal striatum (caudate-putamen). Degeneration of nigrostriatal neurons causes Parkinson's disease. The mesolimbic pathway originates from the midbrain ventral tegmental area and innervates the olfactory tubercle, the ventral striatum (nucleus accumbens) and parts of the limbic system. This pathway is implicated in the psychomotor effects generated by drugs of abuse including cocaine and methamphetamine (Koob, 1992; Wise, 1996). The mesocortical pathway arises from the ventral tegmental area and innervates different regions of the frontal cortex. Finally, the tuberoinfundibular pathway arises from cells of the periventricular and arcuate nuclei of the hypothalamus (Fig 13).

Dopamine is not a simple excitatory or inhibitory neurotransmitter, but rather a neuromodulator that alters the responses of target neurons to other neurotransmitters, and that can alter synaptic plasticity. In this regard, dopamine (as do other monoamines) does not mediate fast synaptic transmission but modulates it by triggering slow-acting effects through signaling cascades.

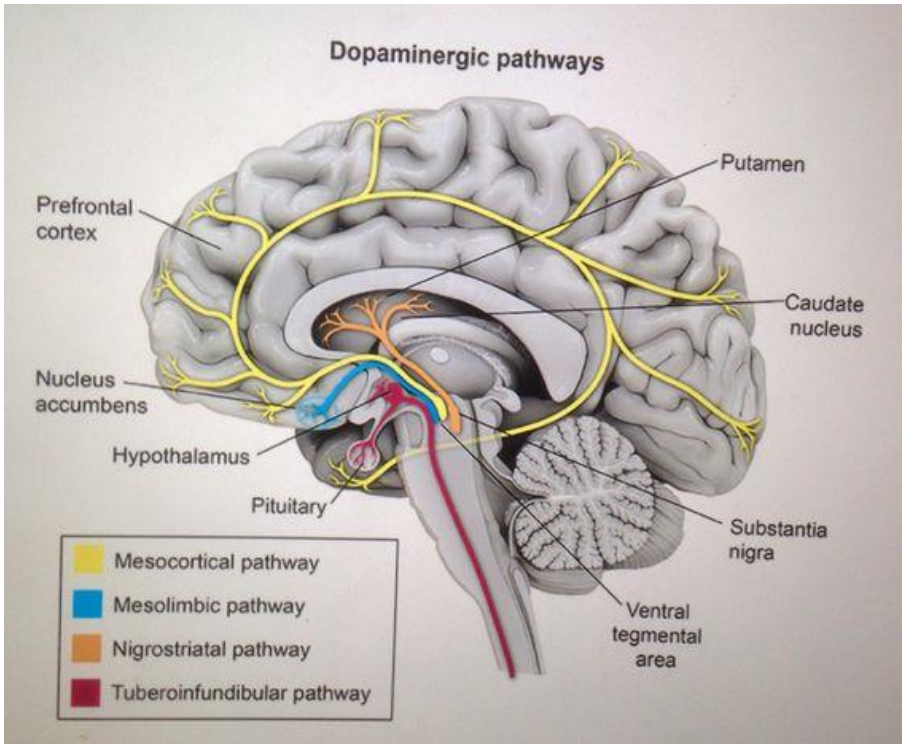

Figure 13. Dopaminergic pathways in the brain.

The dopaminergic systems have been the focus of much research over the past 50 years, mainly because several pathological conditions such as Parkinson's disease (PD), schizophrenia, Tourette's syndrome, attention-deficit hyperactivity disorder (ADHD), Huntington's disease, substance use disorders (SUD) and Restless leg syndrome (RLS; see section 3.5), have been linked to a dysregulation of dopaminergic transmission. Parkinson's disease (PD) originates from a loss of striatal dopaminergic innervations in the brain (Ehringer and Hornykiewicz, 1960). Less straightforward evidence, such as the psychotomimetic effect of dopaminergic drugs and the fact that almost all of the clinically effective antipsychotics block D_2 dopamine receptors, has provided a basis for the dopaminergic hypothesis of schizophrenia (Snyder et al., 1970; Creese et al., 1976; Seeman et al., 1976; Carlsson et al., 2001). Dopamine dysregulation is expected to occur in ADHD and Tourette's syndrome (Mink, 2006; Swanson et al., 2007; Gizer et al., 2009). In Huntington's disease, the selective vulnerability of neurons in the striatum, where the highest concentration of dopaminergic innervation exists, suggests an important role of dopamine in the pathogenesis of this disorder (Jakel and Maragos, 2000; Cyr et al., 2006). The abnormal plasticity of reward

mechanisms that has been shown to be associated with drug abuse and addiction strongly suggests that dopamine plays a crucial role in this pathological condition (Volkow et al., 2003; Bonci et al., 2003; Everitt and Robbins, 2005; Hyman et al., 2006; Di Chiara and Bassareo, 2007; Koob and Volkow, 2010; Sulzer, 2011; Koob and Volkow, 2016). A role for abnormal dopaminergic signaling has also been suggested for a host of other brain disorders, such as bipolar disorder, major depression, dyskinesias, and various somatic disorders, including hypertension and kidney dysfunction (Missale et al., 1998; Aperia, 2000; Carlsson, 2001; Iversen and Iversen, 2007). Hundreds of pharmacologically active compounds that interfere with dopamine receptor functions at the level of ligand binding have been developed, and many of these compounds have been used for clinical applications in the treatment of these and other disorders.

3.3. Characteristics and structure of dopamine receptors

The physiological actions of dopamine are mediated by five distinct but closely related G protein-coupled receptors that are divided into two major groups: The D_1-like, which comprises D_1 and D_5, and D_2-like, including D_2, D_3 and D_4 receptors. D_1-like receptors produce an increase of cAMP levels via Gαs/olf which stimulates adenylyl cyclase (AC) and their localization is mostly postsynaptic (Civelli et al., 1993). D_2-like receptors inhibit AC via Gαi/o coupling and decrease cAMP in the cells. In addition, D_2-like receptors activate K^+ channels and reduce Ca^{2+} entry through voltage-gated channels (Nicola et al., 2000). D_2-like receptors are expressed both postsynaptically on dopamine target cells and presynaptically on dopaminergic neurons (Sokoloff et al., 2006; Rondou et al., 2010).

Analysis of the dopamine receptor structure has revealed considerable homology between members of the same family. The D_1 and D_5 dopamine receptors are 80% homologous in their transmembrane domains, whereas the D_3 and the D_4 dopamine receptors are 75% and 53% homologous, respectively, with the D_2 receptor. Whereas the NH_2-terminal domain has a similar number of amino acids in all of the dopamine receptors, the COOH-terminal for the D_1-like receptors is seven times longer than that for the D_2-like receptors (Gingrich and Caron., 1993; Missale et al., 1998) (see Fig 14). They all contain a cysteine residue (conserved in

GPCRs) that has been demonstrated to be palmitoylated to anchor the cytoplasmic tail of several GPCRs (Ovchinnikov et al., 1988; O'Dowd et al., 1989; Ng et al., 1994; Grunewald et al., 1996). Finally, D_2-like receptors have a long IC3, whereas D_1-like receptors contain a short IC3, a common feature for Gi- and Gs-coupled receptors, respectively (reviewed in Civelli et al., 1993; Gingrich and Caron, 1993; and O'Dowd, 1993).

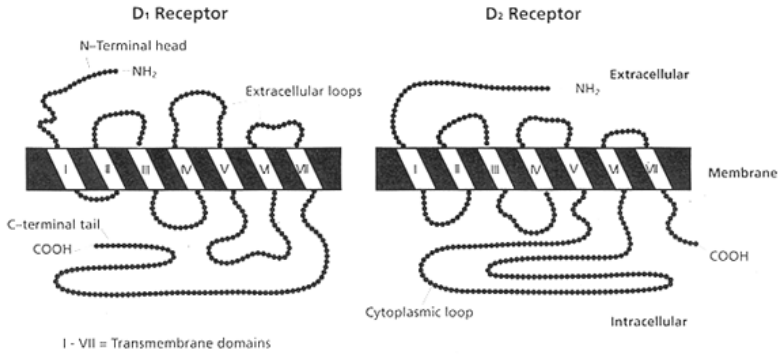

Figure 14. Dopamine D_1 and D_2 receptors structure. Dopamine D_1-like receptors have a very long C-terminal domain and a short IC3 whereas D_2-like receptors have a short C-terminal domain and a very long IC3. *Extracted from Crocker, 1994.*

D_1-like and D_2-like dopamine receptors are also different at the level of genetic structure, primarily in the presence of introns in their coding sequence. The D_1 and D_5 dopamine receptor genes do not contain introns in their coding region, but the genes that encode D_2-like receptors have several introns. Therefore, the genetic organization of the D_2-like receptors provides the basis for the generation of receptor splice variants. For example, the alternative splicing of an 87-base-pair exon of the D_2 dopamine receptor leads to the generation of two major D_2 dopamine receptor variants called D_{2S} (D_2-short) and D_{2L} (D_2-long) (Giros et al., 1989; Monsma et al., 1989). These two alternatively splicing isoforms differ in the presence of an additional 29 amino acids in the third intracellular loop. Interestingly, D_{2S} has been shown to be mostly expressed presynaptically and to be mostly involved in autoreceptor functions, whereas D_{2L} seems to be predominantly a postsynaptic isoform (Usiello et al., 2000; De Mei et al., 2009). Splice variants of the D_3 dopamine receptor have also been described, and some of the encoding proteins have been shown to be essentially nonfunctional (Giros et al., 1991).

The D_4 receptor gene contains quite a large number of polymorphisms in its coding sequence (LaHoste et al., 1996). The most extensive polymorphism is found in exon 3 in a region that codes for the third intracellular loop (IC3) of the receptor (Van Tol, 1992). This polymorphism consists of a variable number of tandem repeats (VNTR), in which a 48-base pair sequence exists as a 2- to 11-fold repeat (Wang et al., 2004). The three most common variants in humans contain 2, 4 and 7 TR and code for a D_4 receptor with 2, 4 and 7 repeats of proline-rich sequence of 16 amino acids ($D_{4.2}$, $D_{4.4}$ and $D_{4.7}$ receptor). A previous study reported *in vitro* evidence for blunted MAPK signaling in striatal slices from mice containing the humanized $D_{4.7}$ in its genome compared to WT D_4, as well as low ability of $D_{4.7}$ to interact with D_2 dopamine receptors in transfected cells (González et al., 2012). Other groups, also suggested that the $D_{4.7}$ variant could be less efficient at inhibiting adenylyl cyclase (Asghari et al., 1995; Jovanovic et al., 1999).

The DRD4 gene with 4 TR constitutes the most frequent variant, with a global allelic frequency of 64%, followed by the variants with 7 TR (21%) and 2 TR (8%) (Chang et al., 1996). However, there are considerable differences in allele frequencies among the different populations. For example, $D_{4.7}$ only appears occasionally (<1%) in several Asian populations, whereas the $D_{4.2}$ allele is more frequent (up to 18%) in Asia than globally (8%). Interestingly, there is evidence showing that seven-repeat alleles are at least five to ten times younger than the common four-repeat allele, but nevertheless have increased in frequency in human populations by positive selection (Ding et al., 2002) (see Fig 15). DRD4 polymorphic variants have been suggested to be associated with numerous behavioral individual differences and neuropsychiatric disorders. The most reported association is the link between the variant with seven repeats and ADHD (Faraone et al., 2005; Li et al., 2006; Gizer et al., 2009) and also substance use disorders (SUD) (McGeary et al., 2009; Belcher et al., 2014). Yet, very little is known about the role of the D_4 receptor in the brain and even less about the functional differences between the products of the different polymorphic variants, which should explain their noticeable influence at the behavioral level.

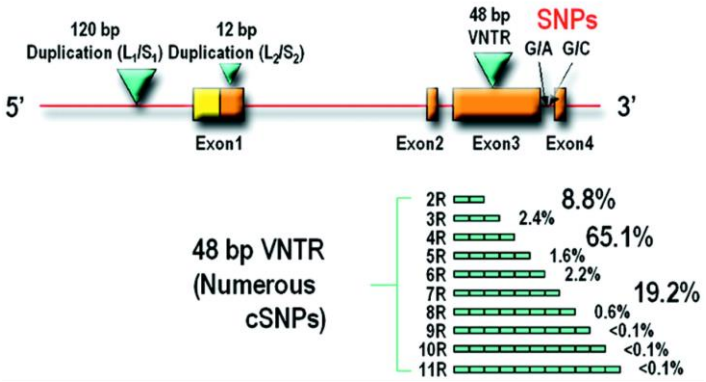

Figure 15. Dopamine D_4 receptor gene polymorphisms. The D_4 gene has an extensive polymorphism in exon 3 that consists of a 48 bp VNTR. The global allelic frequency for D4.2, D4.4 and D4.7 is indicated on the right. *Extracted from Ding et al., 2002.*

3.4. Dopamine receptor expression in the brain

Dopamine receptors have broad expression patterns in the brain and in the periphery. In the brain, D_1 dopamine receptors are the most widespread dopamine receptor and they are expressed at higher levels than any other dopamine receptor (Dearry et al., 1990; Fremeau et al., 1991; Weiner et al., 1991). D_1 has been found at a high density in the nigrostriatal, mesolimbic, and mesocortical areas, such as the caudate-putamen (dorsal striatum), nucleus accumbens, substantia nigra, olfactory bulb, amygdala and frontal cortex, as well as at lower levels in the hippocampus, cerebellum, thalamic areas and hypothalamic areas.

D_5 dopamine receptors are poorly expressed compared to D_1 receptor. They are located at low levels in multiple brain regions, including pyramidal neurons of the prefrontal cortex, the premotor cortex, the cingulated cortex, the entorhinal cortex, substantia nigra, hypothalamus, the hippocampus, and the dentate gyrus. A very low level of expression has also been observed in the MSNs of the caudate nucleus and nucleus accumbens (Choi et al., 1995; Khan et al., 2000; Berlanga et al., 2005).

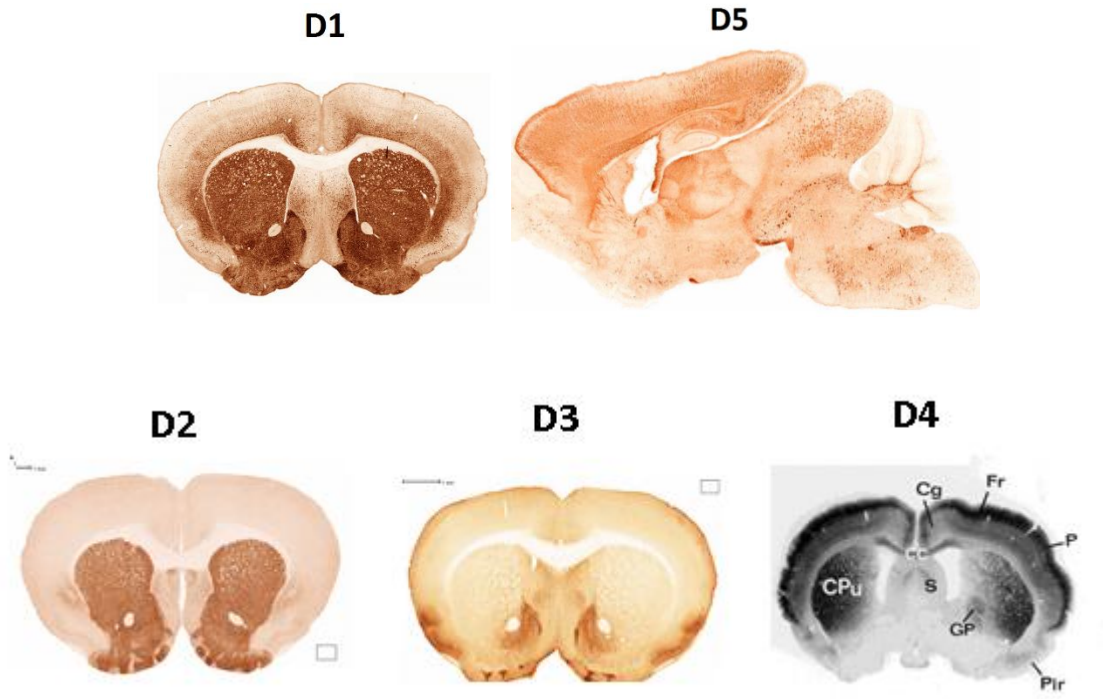

Figure 16. Expression of dopamine receptors in rodent brain. (D1, D2, D3) Coronal sections of D_1-Cre, D_2-Cre or D_3-Cre mouse. Receptor expression was detected by cumulative actions of Cre recombinase at Rosa26 reporter gene. *Extracted from the Gensat atlas.* **(D4)** Coronal section of rat brain. Receptor expression was detected by immunohistochemistry with affinity-purified antibody against D_4. *Extracted and modified from Khan et al., 1998.* **(D5)** Sagittal section of mouse brain. Receptor expression was detected by EGFP fluorescence. *Extracted from Gensat atlas.*

The highest levels of D_2 dopamine receptors are found in the striatum, the nucleus accumbems, and the olfactory tubercle. D_2 receptors are also expressed at significant levels in the substantia nigra, ventral tegmental area, hypothalamus, cortical areas, septum, amygdala, and hippocampus (Missale et al., 1998; Gerfen, 2000; Vallone et al., 2000; Seeman, 2006). The D_3 dopamine receptor has a more limited pattern of distribution, the highest level of expression being observed in the limbic areas, such as in the shell of the nucleus accumbens, the olfactory tubercle, and the islands of Calleja (Sokoloff et al., 1992, 2006; Missale et al., 1998). At significantly lower levels, the D_3 dopamine receptor is also detectable in the striatum, the substantia nigra pars compacta, the ventral tegmental area, the hippocampus, the septal area, and in various cortical areas (Cortés et al., 2016). Low levels of D_4 dopamine receptor have been found in the basal ganglia; in contrast, this receptor appears

to be highly expressed in the frontal cortex, amygdala, hippocampus, hypothalamus, globus pallidus, substantia nigra pars reticulata, and thalamus (Van Tol et al., 1991; O'Malley et al., 1992; Missale et al., 1998; Rondou et al., 2010). In the prefrontal cortex, D_4 is localized in GABAergic interneurons and in glutamatergic pyramidal neurons, including their striatal projections (Tarazi et al., 1998; Svingos et al., 2000; Lauzon and Laviolette, 2010). Interestingly, D_4 and D_2 receptors seem to be colocalizing in the pyramidal glutamatergic neurons of the prefrontal cortex and in their striatal terminals (Gaspar et al., 1995; Tarazi et al., 1998; Svingos et al., 2000; Lauzon and Laviolette, 2010; González et al., 2012). This topic will have important implications for the third part of the results section.

3.5. Neuronal functions of dopamine receptors

The striatum is a major component of the basal ganglia (Kase, 2001) which are located in the telencephalon and consist of several interconnected nuclei: the striatum, external globus pallidus (Gpe), internal globus pallidus (Gpi), substantia nigra (SN), and the subthalamic nucleus (STN) (Fig 17). In primates, the striatum can be divided by the internal capsule into the medially located caudate nucleus and the laterally positioned putamen. The putamen (dorsomedial striatum) receives inputs primarily from the association cortex (Goldman et al., 1977; Ragsdale et al., 1981) and the caudate nucleus (dorsolateral striatum) receives inputs from sensorimotor cortex (Kunzle et al., 1975; Liles et al., 1985). The ventral striatum, or nucleus accumbens, represents a third subdivision of the striatum (Nicola, 2007) and receives glutamatergic inputs from frontal cortex and limbic regions (Brog et al., 1993). However, the dopaminergic innervation of the ventral striatum derives from the ventral tegmental area, a separate midbrain nucleus adjacent to the substantia nigra parts compacta, SNc (Fields et al., 2007). The dorsal striatum (caudate-putamen) is implicated in execution of learning and complex motor behavior, the ventral striatum (nucleus accumbens) participates in motivation, reward and reinforcement learning and plays an important role in addiction (Koob, 1992; Hyman et al., 2006; Iversen and Iversen, 2007; Everitt and Robbins, 2016).

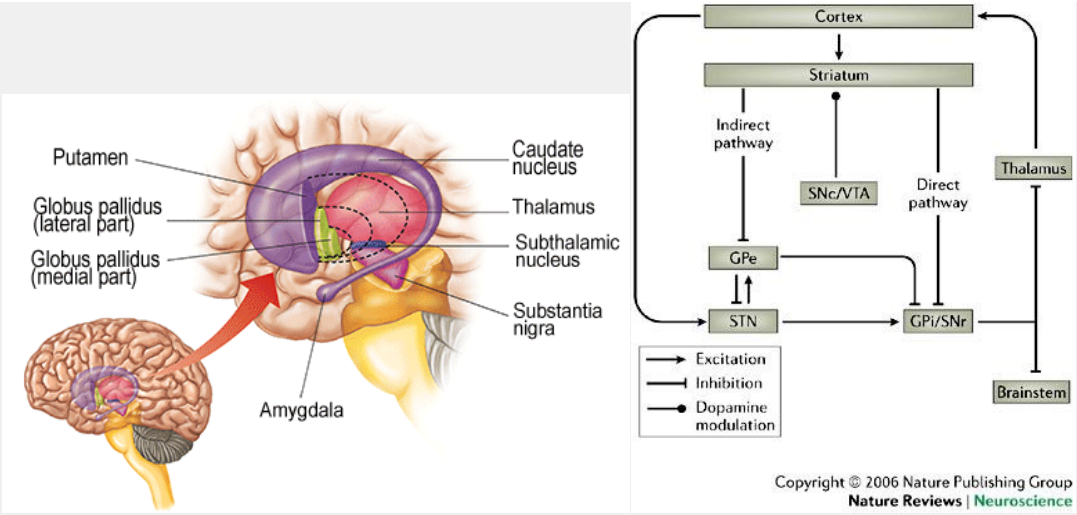

Figure 17. Basal ganglia anatomy in human brain and schematic representation of basal ganglia circuitry. GPe, external globus pallidus; GPi, internal globus pallidus; STN, subthalamic nucleus; SNr, substantia nigra pars reticulata. *Extracted from Yin and Knowlton, 2006.*

Early investigations more than a century ago, pointed to a complex relationship between the striatum and movement. An anatomical scheme for understanding the control of movement by the striatum was defined in the late 1980s (Penney et al., 1986; Albin et al., 1989; DeLong, 1990). This scheme recognized that the majority of dorsal striatal neurons are medium spiny neurons (MSNs), of which there are two distinct classes, termed the "direct" and the "indirect" pathway (Alexander and Crutcher, 1990; DeLong, 1990; Gerfen et al., 1990; Graybiel et al., 1994 Le Moine and Bloch, 1995; Obeso et al., 2008) (See Fig 17). These populations exhibit distinct neurochemical expression patterns and anatomical projection targets. Direct pathway MSNs express D_1 receptor and project to the internal globus pallidus and substantia nigra pars reticulata (SNr), whereas indirect pathway MSNs express D_2 receptors and project indirectly to the SNr by way of the external globus pallidus (GPe) and subthalamic nucleus (STN). Based on this anatomy, it has been demonstrated in many studies that activation of direct pathway striatal neurons promoted movement whereas activation of the indirect pathway inhibits movement (Sano et al., 2003; Durieux et al., 2009; Kravitz et al., 2010). Striatum has high levels of dopamine and this catecholamine is the one that controls and regulates movement. Due to the differential expression of D_1 and D_2 dopamine receptors in the direct and indirect pathway, respectively, dopamine affects the neurons of each

pathway differently (Gerfen et al., 1990). The dopamine D_1 receptor is coupled to Gαs, which activates adenylyl cyclase and increases intracellular cAMP (Kebabian and Calne, 1979; Stoof and Kebabian, 1981; Gerfen, 2000). This increase in cAMP results in multiple intracellular effects that increase the excitability of direct pathway neurons. In contrast, the dopamine D_2 receptor is coupled to Gαi, which inhibits adenylyl cyclase and decreases intracellular cAMP (Kebabian and Calne, 1979; Stoof and Kebabian, 1981; Gerfen, 2000). This decrease in cAMP is believed to decrease the excitability of indirect pathway neurons. Therefore, dopamine exerts a fine-tuning effect on both direct and indirect pathway allowing controlled movement. In addition, regulation of cAMP/PKA signaling by dopamine receptors may directly or indirectly regulate the induction of striatal synaptic plasticity (Kreitzer and Malenka, 2008; Surmeier et al., 2009). When striatal dopamine levels decrease in Parkinson's disease, activity in the direct pathway is believed to decrease and activity in the indirect pathway is believed to increase (Albin et al., 1989; Graybiel et al., 2000; Obeso et al., 2000, 2008; Smith and Villalba, 2008). On the other hand, excess of dopaminergic stimulation would lead to hyperkinesia. In Huntington's disease, hyperkinetic choreic movements are due to a gradual disappearance of the contribution of the indirect inhibitory pathway followed by the degeneration of the direct pathway and the nigrostriatal neurons (Glass et al., 2000). Therapies aimed at rebalancing the activity in these pathways form the basis for most therapeutic interventions (Filion et al., 1991; Kanda et al., 1998; Dostrovsky et al., 2000; Levy et al., 2001; Baron et al., 2002; Matsubara et al., 2002; Jenner, 2003; Rose et al., 2007; Kravitz et al., 2010).

Another important aspect of dopamine receptors is their role as autoreceptors. Presynaptic localized autoreceptors generally provide an important negative feedback mechanism that adjusts neuronal firing rate, synthesis, and release of the neurotransmitter in response to changes in extracellular neurotransmitter levels (Wolf and Roth., 1990; Missale et al., 1998; Sibley 1999). Activation of presynaptic D_2-like autoreceptors generally causes a decrease in dopamine release that results in decreased locomotor activity, whereas activation of postsynaptic receptors stimulates locomotion. It should be noted that the splice variants of the D_2 dopamine receptor, D_{2L} and D_{2S}, seem to have different neuronal distributions, D_{2S} being predominantly presynaptic and D_{2L} postsynaptic. Therefore, the varying roles of the postsynaptic and presynaptic D_2 dopamine receptors are probably determined by the

different contributions of these isoforms (Usiello et al., 2000; De Mei et al., 2009). D_3 dopamine receptors seem to exert a moderate inhibitory action on locomotion either by acting as autoreceptor or through the involvement of postsynaptic receptor populations (Sibley, 1999, Joseph et al., 2002). The roles of D_4 and D_5 dopamine receptors in locomotor activity seem to be minimal. However, it is clear that the activation of both the postsynaptic D_1- and D_2-like dopamine receptors is necessary for the full manifestation of locomotor activity (White et al., 1988).

Many other vital functions besides locomotor activity depend on the activation of brain dopamine receptors. D_1, D_2, and, to a lesser degree, D_3 dopamine receptors are critically involved in reward and reinforcement mechanisms. Multiple studies have shown that pharmacological and genetic approaches that alter dopamine receptor function result in a significant modulation of the response to natural rewards and addictive drugs. Thus, dopamine receptors remain an important topic of interest in drug addiction research (Missale et al., 1998; Hyman et al., 2006; Sokoloff et al., 2006; Di Chiara and Bassareo, 2007; De Mei et al., 2009; Koob and Volkow, 2010). Both D_1 and D_2 dopamine receptors seem to be critical for learning and memory mechanisms, such as working memory, that are mediated primarily by the prefrontal cortex (Goldman-Rakic et al., 2004; Xu et al., 2009). At the same time, D_3, D_4 and potentially, D_5 dopamine receptors seem to have a modulatory influence on some specific aspects of cognitive functions (Missale et al., 1998; Sibley, 1999; Sokoloff et al., 2006; Rondou et al., 2010). Other functions are mediated in part by various dopamine receptor subtypes in the brain such as affect, attention, impulse control, decision making, motor learning, sleep, reproductive behaviors, and regulation of food intake (Missale et al., 1998; Di Chiara and Bassareo, 2007; Iversen and Iversen, 2007; Koob and Volkow, 2010; Rondou et al., 2010). In general, the specific physiological roles played by D_3, D_4 and D_5 dopamine receptors in the brain are not well known. However, a study recently published led to the demonstration of a significant role of D_4 receptor and one of its variants ($D_{4.7}$), in the modulation of corticostratal glutamatergic transmission that could have important implications for the understanding of neuropsychiatric disorders such as ADHD and SUD (Bonaventura et al., 2016).

3.6. Restless leg syndrome

Until the end of the 20th century, restless legs syndrome (RLS), also known as Willis-Ekbom syndrome, was a relatively unknown disorder. Convincing evidence now indicates that it is a common disorder with a prevalence of 1-5% (Allen et al., 2003, 2014; Szentkirályi et al., 2014), more frequent in females, and good therapeutic options are available. The condition has been clearly established as an important element of clinical practice in the field of movement disorders, and also in the field of sleep disorders: RLS symptoms are circadian in nature (Trenkwalder et al., 1999) and cause major problems for patients in the evening and at night.

The restless leg syndrome has been described as an idiopathic sensorimotor disorder characterized by an urge to move the limbs which is usually associated with unpleasant sensations. It has no apparent cause although it has been often associated with iron deficiency, pregnancy, or end-stage renal disease. However, the syndrome could also be described as a complex disorder with underlying genetic or environmental components, or both. Onset in childhood implies a strong genetic component for the syndrome (Winkelmann et al., 2002), although the age of onset in general is known to vary widely, from childhood to over 80 years of age (Walters et al., 1996; Winkelmann et al., 2000). Clinical experience shows severe restless legs syndrome to be mostly a chronic progressive disorder that, once started, needs lifelong treatment.

RLS is usually managed with pharmacological treatment. In the 1990s, Trenkwalder et al. (1995), used a combination of L-DOPA with a DOPA decarboxylase inhibitor (DDCI) to treat restlessness, sleep disturbance and sensory and motor symptoms in patients with RLS, and various dopamine agonists have been used since (reviewed in Scholz et al., 2011; Trenkwalder et al., 2015). Therapy with non-ergot-derived dopamine agonists is currently recommended in several guidelines as the first-line therapy for RLS (Aurora et al., 2012; García-Borreguero et al., 2012). Oral immediate-release pramipexole, oral immediate-release ropinirole and the rotigotine patch are licensed in many countries, including Europe, the USA and Japan (structure shown in Fig 18). Levodopa-benserazide can still be used, but the short half-life of levodopa and its pulsatile mode of action can lead to higher rates of augmentation than do other dopamine agonists (Trenkwalder et al., 2003; Högl et al., 2010, 2011; Oertel et al., 2011; Inoue et al., 2013). Augmentation is the main problem in dopaminergic RLS therapy and can

be defined as a long-term paradoxical worsening of RLS symptoms while on dopaminergic therapy, despite a short-term improvement immediately after drug intake.

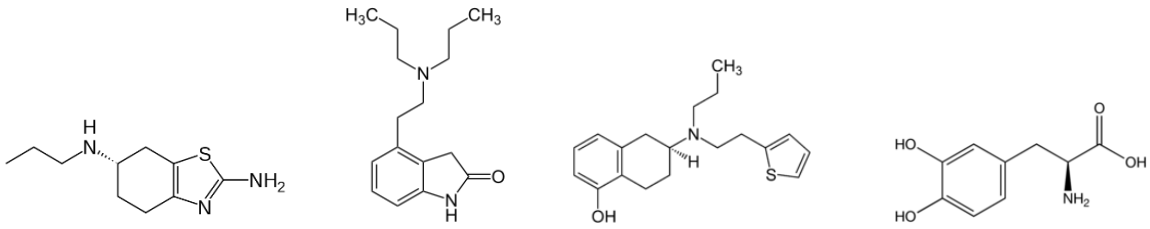

Figure 18. Chemical structure of the dopaminergic ligands used for the treatment of RLS

Other non-dopaminergic therapies are α2δ calcium channel subunit ligands (García-Borreguero et al., 2002; Allen et al., 2014), however only gabapentin enacarbil has been licensed for treatment of RLS in the USA (Kushida et al., 2009ab) and Japan (Inoue et al., 2012, 2013). Opioids were also found to be an effective treatment for severer RLS over 20 years ago (Walters et al., 1993), and one large clinical trial has led to approval of oxycodone-naloxone therapy as a second-choice treatment for RLS in Europe in 2015 (Trenkwalder et al., 2013). Several studies have also been performed to determine the benefit of iron supplementation in both iron-deficient and non-iron-deficient patients with RLS (Grote et al., 2009; Earley et al., 2009; Allen et al., 2011, 2013, Mehmood et al., 2014). Results have been inconsistent, but might improve when patient selection is optimized.

In general, all currently know therapies for RLS seem to lose effectiveness over time in many patients with severe RLS. Current treatment guidelines, which are based on long-term studies, recommend starting treatment with either a dopamine receptor agonist or a α2δ ligand as the first-line of treatment for most RLS patients, the choice of agent depending on the severity of the symptoms, cognitive status, history, comorbid conditions and licensing of drugs in a given country (García-Borreguero et al., 2013).

At the moment the pathophysiology of RLS is not totally known. However, the fact that dopaminergic ligands are useful for the treatment of RLS provides the basis for the current, widely held view that RLS involves dopaminergic dysfunction. Most reported studies focus on

the D3R as a possible main target of pramipexole, ropinirole and rotigotine although some papers show the relatively high affinity of D2R and D4R for these ligands as well (Millan et al., 2002; Wood et al., 2015). As previously said, D4R and D2R may form heteromers in the brain, probably in the cortico-striatal neuronal terminals. This opens a new possibility for the study of these ligands in the frame of heteromers. This part will be discussed in the third chapter of the results section.

4. Adrenergic receptors

4.1. Adrenergic system in the brain

In 1913 adrenaline was first isolated and its effects on vasodilation and constriction were demonstrated. However, it wasn't until 1950 that the function of the catecholamines (epinephrine and norepinephrine) as neurotransmitters in the brain was stablished.

Norepinephrine is synthesized and released by the central nervous system and also by the sympathetic nervous system (a division of the autonomic nervous system). In the brain, norepinephrine is mainly produced in the locus coeruleus (LC). In the sympathetic nervous system, norepinephrine is used as a neurotransmitter by sympathetic ganglia located near the spinal cord or in the abdomen, and it is also released directly into the bloodstream by the adrenal glands as sympathetic effector organs.

The general function of norepinephrine is to mobilize the brain and body for the so-called "fight-or-flight response". Norepinephrine release is lowest during sleep, rises during wakefulness, and reaches much higher levels during situations of stress or danger. In the brain, norepinephrine increases arousal and alertness, promotes vigilance, enhances formation and retrieval of memory, and focuses attention; it also increases restlessness and anxiety. In the rest of the body, norepinephrine increases heart rate and blood pressure, triggers the release of glucose from energy stores, increases blood flow to skeletal muscle, reduces blood flow to the gastrointestinal system, and inhibits voiding of the bladder and gastrointestinal motility.

The locus coeruleus was the first modulatory system to be delineated anatomically (Sano, 1942; Araki, 1947; Russel, 1955; Maeda, 2000) and specified neurochemically (Dahlstom and Fuxe, 1964). The tiny LC is comprised of only 1500 neurons in the rat (around 15000 in humans/hemisphere) and is situated deep in the pons. It receives afferents from the midbrain and brainstem conveying information about visceral and sympathetic nervous system function as well as pain and threat. It also receives inputs from the forebrain such as the hypothalamus, the amygdala and the prefrontal cortex that provide complex emotional,

homeostatic and cognitive information. The LC also receives projections from various neuromodulatory brain regions including the ventral tegmental area (dopamine) and dorsal raphe (serotonin). Together, these afferents connections allow for modulation of the LC neural processing by basic sensory and visceral experiences as well as regulation by top-down influences from forebrain structures conveying highly processed cognitive/emotional information (Berridge and Waterhouse, 2003; Aston-Jones and Cohen 2005; Sara and Bouret, 2012).

Despite the small number of neurons in the LC, it projects broadly to most forebrain regions as well as some midbrain and brainstem nuclei and the cerebellum and spinal cord (Berridge and Waterhouse, 2003; Aston-Jones and Cohen, 2005; Sara and Bouret, 2012; Valentino and Van Bockstaele, 2008). Related to learning and memory, the LC sends strong efferent projections to the amygdala and mPFC (Fallon et al., 1978; Arnsten, 1984) (Fig 18).

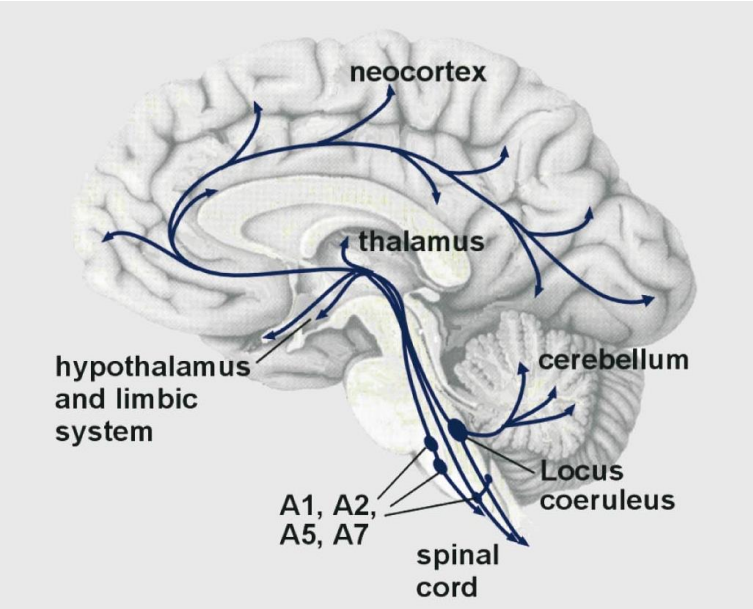

Figure 18. Noradrenergic system in the brain. The noradrenergic neurons of the locus coeruleus project to the limbic and cortical regions, and to the thalamus, cerebellum, and spinal cord. The noradrenergic neurons of cell groups A1, A2, A5, and A7 project to more restricted regions and they play an important role in autonomic functions. *Modified from Fuchs and Flugge, 2004.*

Noradrenergic neurons in the LC play a critical role in many functions including physiological responses to stress and panic, learning and memory. A large body of literature implicates

noradrenaline also in cellular excitability, synaptic plasticity and long-term potentiation (Harley, 1987, 2007). An equally large number of studies have demonstrated the role of noradrenaline in gating and tuning sensory signals in the thalamus and the cortex (Berridge and Waterhouse, 2003). Pharmacological studies have provided evidence that noradrenaline, interacting with other neuromodulators and hormones, modulates memory formation, mainly through actions in the amygdala and the hippocampus (Cahill and McGaugh, 1996). Other pharmacological approaches have revealed a noradrenergic influence in frontal cortical regions that are engaged in attention and working memory functions (Arnsten and Li, 2005; Robbins and Roberts, 2007). In addition, electrophysiological studies in behaving primates and rodents have shown a clear relationship between activity in the locus coeruleus neurons and cognitive behaviors (Aston-Jones and Cohen, 2005; Yu and Dayan, 2005; Bouret and Sara, 2005).

4.2. Characteristics and structure of adrenergic receptors

Both norepinephrine and epinephrine transmit their biological signals via GPCR to regulate a large array of cellular functions (Hoffman and Lefkowitz, 1980). These receptors are mostly localized in the plasma membrane of noradrenergic neurons and neuronal and non-neuronal target cells.

Adrenoceptors were originally divided into two different classes, α-adrenoceptors and β-adrenoceptors. This distinction of receptor subtypes was introduced more than 60 years ago by Ahlquist who observed opposing effects of catecholamine derivatives in smooth muscle cells (Ahlquist, 1948). He suggested that the excitatory effects were mediated by α-adrenoceptors, whereas the inhibitory effects were mediated by β-adrenoceptors (Ahlquist, 1948). In addition, α-adrenoceptors showed higher affinity for norepinephrine and epinephrine than for isoprenaline (a synthetic catecholamine) whereas β-adrenoceptors have higher affinity for isoprenaline than for norepinephrine. Subsequently, further subtypes were proposed based on physiological and pharmacological experiments. Molecular cloning rapidly confirmed the subtype diversity of adrenoceptors. In 1986, the β_2-adrenoceptor was the first

GPCR to be cloned (Dixon et al., 1986), closely followed by the α_2-adrenoceptor, which was purified and cloned from human platelets (Kobilka et al., 1987). Rapidly nine adrenoceptors were identified by molecular cloning (Bylund et al., 1994); three α_1-adrenoceptors (α_{1A}, α_{1B}, α_{1D}), three α_2-subtypes (α_{2A}, $\alpha_{2B,}$ α_{2C}), and three β-adrenoceptors (β_1, β_2, β_3).

All adrenoceptor subtypes belong to the GPCR family so upon binding of the endogenous activators (epinephrine and norepinephrine), adrenoceptors undergo a conformational change that leads to the activation of a heterotrimeric G protein. The three groups of adrenoceptors couple to and activate only certain G protein subtypes leading to different intracellular changes. α_1-adrenoceptors are coupled to Gq/11, which increases activates PLC and increases intracellular inositol triphosphate (IP_3) and Ca^{2+} concentrations. α_2-adrenoceptors mostly mediate their intracellular effects via Gi/o which inhibits adenylyl cyclase producing a decrease in cAMP. In addition, Gβγ subunits released from activated Gi/o proteins are important regulators of neuronal function by inhibiting neuronal Ca^{2+} channels and activating G protein-coupled inwardly-rectifying potassium channels (GIRK) and mitogen-activated kinases (MAPK) ERK1/2 (Cussac et al., 2001). Finally, β-adrenoceptors couple to Gs/olf which leads to activation of adenylyl cyclase and increase of cAMP in the cell.

Another important aspect of signaling, as explained earlier, is the receptor phosphorylation mediated by GRKs that leads to desensitization. Adrenoceptors, like other GPCRs, recruit arrestins which prevents further G protein activation as well as generates a protein scaffold for further signaling processes including MAPK, AKT and PI3K.

One important distinguishing feature among α_2-receptor subtypes is their pattern of subcellular distribution and agonist-induced trafficking (Saunders and Limbird, 1999). The α_{2A}-subtype is efficiently targeted to the cell surface membrane and only a small proportion of receptor internalizes in response to agonist activation (von Zastrow et al., 1993) α_{2B}-receptors are rapidly and reversibly internalized after agonist simulation, whereas α_{2C}-adrenoceptor reside in an intracellular compartment in many cell types and are not effectively targeted to the plasma membrane (Daunt et al., 1997; Hurt et al., 2000).

Within the 7 transmembrane domains the adrenergic receptors contain considerable amino acids sequence identity. The highest identity (~70%) is usually found among members of the same subfamily (i.e. α_{1A}, α_{1B} and α_{1C}; β_1, β_2 and β_3). Within members of the adrenergic

receptor family (i.e. β vs α_1 or α_2), the identity within the transmembrane segments falls to about 45%. The regions of greatest diversity even among related receptor subtypes are the extracellular amino terminus, the third cytoplasmic loop as well as the carboxyl terminal (Roth et al., 1991).

The primary structures of the nine adrenergic receptor subtypes display similar characteristic features: a single polypeptide chain from 400 to over 500 residues long comprising amino- and carboxy-terminal regions variable both in length and sequence, and three intracellular, three extracellular and seven well conserved hydrophobic transmembrane domains. The α_2 receptor subtype C-terminal regions are shorter than those of the β and much shorter than those of the α_1 subtypes (Fig 19). This goes in line with the observation that Gs-coupled receptors (β) or Gi/o (α_2) generally have short intracellular loops 3 and C-terminal segments, whereas receptors involved in other effector systems such as phospholipase C (α_1) have longer sequences in these regions. The human α_{2B} thus has a 23-residue C-terminus, whereas the human α_{1B} C-terminal region is 167 long (Strosberg, 1993).

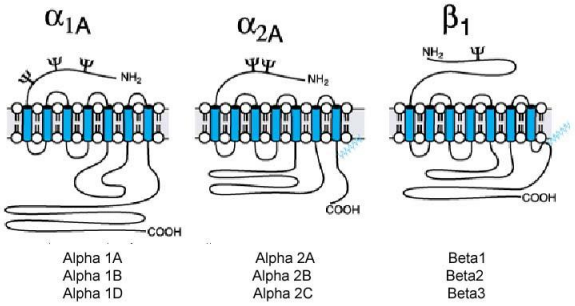

Figure 19. Structure of adrenergic receptors

4.3. Expression of adrenergic receptors in the brain

α_1-Adrenoceptor is found throughout the mesocorticolimbic system with high levels in the rat striatum, ventral tegmental area and susbtantia nigra (Rommelfanger et al., 2009). Specifically, α_{1A} was also found in cortex, hippocampus, hypothalamus, midbrain, cerebellum and spinal cord (Papay et al., 2006).

When looking into the tissue distribution of α_2-adrenoceptors, the lack of selective ligands for α_2-subtypes prevents their proper study by autoradiography or binding. Nevertheless, the use of [^{3}H]rauwolscine, which preferably binds to the α_{2C} subtype, has helped to study the distribution of this receptor subtype in some detail, especially in rodent tissues where this radioligand offers better discrimination of α_{2C} *versus* α_{2A} compared to the situation in humans (reviewed in MacDonald et al., 1992). One example of this is illustrated in Fig 20 where Holmberg et al. (2003) compared the binding of [^{3}H]RX821002 (a non-selective α_2-adrenoceptor ligand) and [^{3}H]rauwolscine (α_{2C} preferred ligand) to account for the expression of α_2 *versus* α_{2C}, respectively. Fig 20 shows high [^{3}H]RX821002 binding density in the cortex and within the basal ganglia, especially in the nucleus accumbens but also in caudate-putamen nucleus, islands of Calleja, olfactory tubercle and ventral pallidum. Moderate levels of [^{3}H]rauwolscine binding were observed in the caudate-putamen nucleus and the olfactory tubercle. Sparse binding was seen over the nucleus accumbens, the islands of Calleja, the ventral pallidum and the cortex.

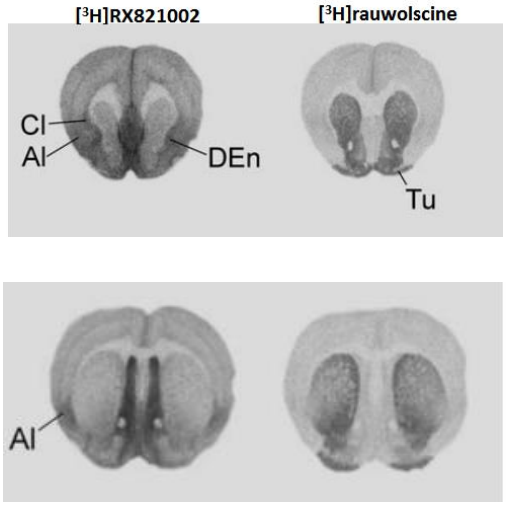

Figure 20. [^{3}H]RX821002 and [^{3}H]rauwolscine (α_{2C}-prefering ligand) binding in forebrain structures at the levels of the nucleus accumbens (upper panel) and caudate-putamen (lower panel). Cl, claustrum: Al, agranular insular cortex; Den, dorsal endopiriform nucleus; Tu, olfactory tubercle. *Modified from Holmberg et al., 2003.*

Another set of experiments by Fagerholm et al. (2004) are shown in Fig 21 where the binding of the non-selective α_2-adrenoceptor ligand [^{3}H]RX821002 is shown in a coronal section of a mouse. The signal is dramatically reduced in α_{2A}-KO mouse confirming the predominance of α_{2A} compared to other α_2-adrenoceptor subtypes, especially in the cortex. Another study by Holmberg et al. (1999) is shown in Fig 22 where they map the distribution of α_{2C} by using the α_{2C}-preferred ligand [^{3}H]rauwolscine. The Fig 22 shows the high expression of α_{2C} in the striatum, olfactory tubercle and substantia nigra.

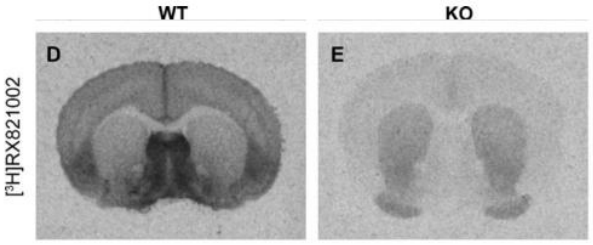

Figure 21. Autoradiography images of WT and α_{2A}-KO coronal mouse brain sections at the level of the caudate-putamen, labelled with [^{3}H]RX821002. *Extracted from Fagerholm et al., 2004*

Another technique used to map the distribution of α_2-adrenoceptor subtypes is *in situ* hybridization. This technique confirms the results just described in which the α_{2A} is the predominant α_2-adrenoceptor in the brain whereas α_{2C} has a more restricted expression pattern and is highly enriched in the striatum (Fagerholm et al., 2008; Lehto et al., 2015), although with a lower density than α_{2A} (Ordway et al., 1993; Uhlen et al., 1997). Finally, the α_{2B} subtype is mostly restricted to the thalamus (Nicholas et al., 1993; Scheinin et al., 1994).

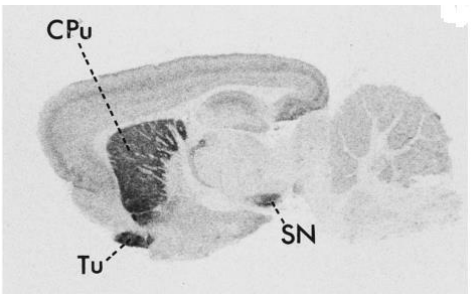

Figure 22. Autoradiographic localization of [^{3}H]rauwolscine binding sites in the rat brain. Sagittal section showing areas with high level of binding (Tu, olfactory tubercle; SN, substantia nigra; CPu, caudate-putamen). *Extracted from Holmberg et al., 1999.*

β-Adrenergic receptor subtypes have differential expression patterns. β_1-adrenoceptor is found at its highest levels in the heart and brain (Frielle et al., 1987), β_2-adrenoceptor is more widely expressed (Dixon et al., 1987), and β_3-adrenoceptor is found at its highest levels in adipose tissue (Emorine et al., 1989; Summers et al., 1995). In the brain, β_1-adrenoceptor is highly expressed in the cortex (Rainbow et al., 1984; Nicholas et al., 1993), especially in the intermediate layers of the prefrontal cortex (PFC) where thalamic inputs are concentrated (Goldman-Rakic et al., 1990), but also in the thalamus, pineal gland and sympathetic ganglia (Nicholas et al., 1996). β_2-Adrenoceptors are expressed in the olfactory bulb, cerebral cortex, hippocampus, thalamus, hypothalamus, pineal gland and spinal cord (Nicholas et al., 1996). They have also been localized on dendritic spines of PFC pyramidal neurons and on GABAergic interneurons (Aoki et al., 1998). Finally, many β-adrenoceptors are found on glia (Hansson and Ronnback, 1991; Fillenz and Lowry, 1998; Fillenz et al., 1999).

4.4. Neuronal functions of adrenergic receptors

Because of the lack of sufficiently subtype-selective ligands, the unique physiological properties of the adrenoceptor subtypes, for the most part, have not been fully elucidated until recently. However, studies in mice carrying deletions in the genes encoding individual adrenoceptor subtypes have greatly advanced our knowledge regarding the specific functions of these receptors (MacDonald et al., 1997; Rohrer, 1998; Rohrer and Kobilka, 1998; Brette et al., 2004; Philipp and Hein, 2004).

Whereas norepinephrine exerts a wide spectrum of effects in the central nervous system, the contribution of the α_1-adrenoceptor to these neuronal functions is largely unknown. Some studies suggest that the noradrenergic pathway is important for the modulation of behaviors such as reaction to novelty and exploration and suggest that this behavior is mediated, at least partly, through α_{1B}-adrenoceptors (Spreng et al., 2001). Other studies indicate the critical role of α_{1B}-adrenoceptors and noradrenergic transmission in the vulnerability of addiction (Drouin et al., 2002).

In mammalian species, both α_{2A} and α_{2C} receptors seem to be localized mostly postsynaptically, preferentially in GABAergic striatal efferent neurons (Holmberg et al., 1999;

Hara et al., 2010). However, α_2-adrenoceptors can also be expressed presynaptically (autoreceptors) where they work as presynaptic feedback inhibitors of neurotransmitter release (Langer, 1997; Starke, 2001). The term "autoreceptors" is used when the receptor is stimulated by neurotransmitter released from the same neuron. Surprisingly, all three α_2-adrenoceptor subtypes contributed to presynaptic feedback inhibition of noradrenaline release from adrenergic neurons *in vitro* (Trendelenburg et al., 2003). Yet, differences between the three α_2-subtypes and between tissues were identified. Similar to pharmacological predictions, the α_{2A}-subtype was found to be the main inhibitory presynaptic feedback receptor in the sparse striatal noradrenergic terminals (Altman et al., 1999; Hein et al., 1999; Trendelenburg et al., 2003; Ihalainen and Tanila, 2004). In addition to the α_{2A}-subtype, α_{2C} participated in presynaptic regulation in the central nervous system, whereas all three α_2-receptor subtypes served as feedback regulators in peripheral tissues which are innervated by sympathetic nerves (Trendelenburg et al., 2003). In addition to their function as inhibitory autoreceptors, α_2-adrenoceptors can also regulate a number of other neurotransmitters in the central and peripheral nervous system and thus operate as "heteroreceptors" (presynaptic receptors activated by transmitters from neighboring neurons). In the brain, α_{2A} and α_{2C}-adrenoceptors inhibit dopamine release in basal ganglia (Bucheler et al., 2002) and serotonin secretion in mouse hippocampus and brain cortex (Scheibner et al., 2001a). The inhibition of neurotransmitter release has also been linked with the neuroprotective effects of α_2-agonists, which are mostly mediated via the α_{2A}-subtype (Ma et al., 2004ab; Paris et al., 2006). Other functions of α_2 receptors in the brain are analgesia, sedation/hypnosis, processing of sensory information and centrally-mediated cardiovascular control (MacMillan et al., 1996; Lakhlani et al., 1997, Altman et al., 1999; Philipp et al., 2002).

In contrast to the α_2-adrenoceptors, which are important for the control of neurotransmitter release, the β-receptors are mostly known for their role in the regulation of cardiovascular, uterine, and peripheral metabolic functions. An important aspect of β_1 is their expression in medium spiny neurons (Nahorski et al., 1979; Waeber et al., 1991), where the loss of β_1-adrenoceptors in the striatum was reported in the late stages of Huntington's disease (Waeber et al., 1991). In the pineal gland, norepinephrine released from sympathetic nerves controls the circadian rhythm of melatonin synthesis via β-adrenoceptors (Simonneaux and

Ribelayga, 2003) and/or D_4 dopamine receptor heteromers (González et al., 2012). Several studies using mice lacking β_1-, β_2-, or β_3-adrenoceptors indicate that these receptors play a central role in regulating numerous functions of the central nervous system, including the regulation of sympathetic tone, learning and memory, mood and food intake. Norepinephrine acting at β_1-adrenoceptors has been found to be essential for the retrieval of contextual and spatial memory but is not essential for the retrieval of emotional memories (Winder et al., 1999; Murchison et al., 2004). Presynaptic β-adrenoceptors may also play an important role in the regulation of neurotransmitter release (Trendelenburg et al., 2000). In humans, many drugs are currently being used that may interact with neuronal β-adrenoceptors. β-blockers are used to treat chronic migraine, glaucoma, or essential tremor (Hoffman and Lefkowitz, 1996). However, the exact mechanism of action of these drugs in these conditions has not been identified as yet.

5. Biophysical techniques to study protein-protein interactions

Resonance energy transfer (RET) techniques have become experimental methods of choice for measuring constitutive and dynamic protein-protein interactions and interrogating changes in the activity of many biochemical signaling pathways. Over the last decade, the main usage of RET has resided into investigating various protein-protein interactions with major usage in the field of GPCRs (Ayoub and Pfleger, 2010; Ferré et al., 2010; Lohse et al., 2012 for review). The list of publications demonstrating GPCR oligomerization using RET techniques is extensive and expanding rapidly.

The theory of resonance energy transfer (RET) was formulated in 1948 by Theodor Förster (Förster, 1948). This phenomenon is based on a non-radiative energy transfer (dipol-dipol) from a cromophore in an excited state (donor), to a close molecule that absorbs it (acceptor). In FRET (fluorescence resonance energy transfer) both molecules are fluorescent, whereas in BRET (bioluminiscence resonance energy transfer) the donor molecule is an enzyme which becomes bioluminescent upon catalyzation of its substrate (Fig 23).

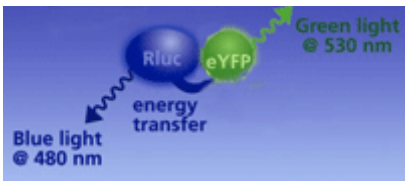

Figure 23. Phenomenon of BRET. Rluc (Renilla luciferase) becomes bioluminescent upon oxidation of its substrate and emits blue light (480 nm) which can excite the YFP (yellow fluorescent protein) and in turn produce emission of green light (530 nm). The BRET ratio is calculated dividing fluorescence between luminescence.

Luminescence is a phenomenon occurring naturally in several marine animals such as in the jellyfish *Aequorea Victoria* or the sea pansy *Renilla reniformis*, of which Rluc was isolated. There are two requirements needed for the energy transfer to take place, the first is that the emission spectrum of the donor and the excitation spectrum of the acceptor overlap. The second requirement is that the distance between donor and acceptor should be equal or below 10 nM (or 100 Å) (Milligan, 2004; Pfleger and Eidne, 2005; Marullo and Bouvier, 2007). Importantly, the efficacy of energy transfer is inversely proportional to the sixth power of the

distance between the donor and acceptor cromophores. The dependence of the energy transfer on the distance between donor and acceptor permits real-time measurements that are both sensitive and specific to the labeling sites of the proteins, thus allowing inference of dynamic structural changes.

BRET has advantage over FRET since it does not require an external illumination to initiate the energy transfer, which may lead to high background noise resulting from direct excitation of the acceptor or photobleaching. BRET experiments can be conducted under conditions that more closely reflect the biochemical environments occurring in living organisms.

BRET technique exists in two variants, BRET^1 and BRET^2 (Fig 24). In BRET^1 the substrate of the luciferase is coelenterazine H which gets oxidized and emits blue light at 480 nm. At this wavelength, the YFP gets excited and in turn emits at 530 nM. In BRET^2 the donor uses Deep Blue C as the substrate emitting light at 400 nM and the acceptor is GFP2 that emits at 510 nM. The advantage of BRET^2 is that there is a bigger separation between the donor and acceptor peaks. The disadvantage is that it has 100 to 300 times lower intensity of emitted light since the luminescence emitted by Rluc using Deep Blue C as substrate experiences a faster signal decay and lower quantum yield than that emitted by coelenterazine H.

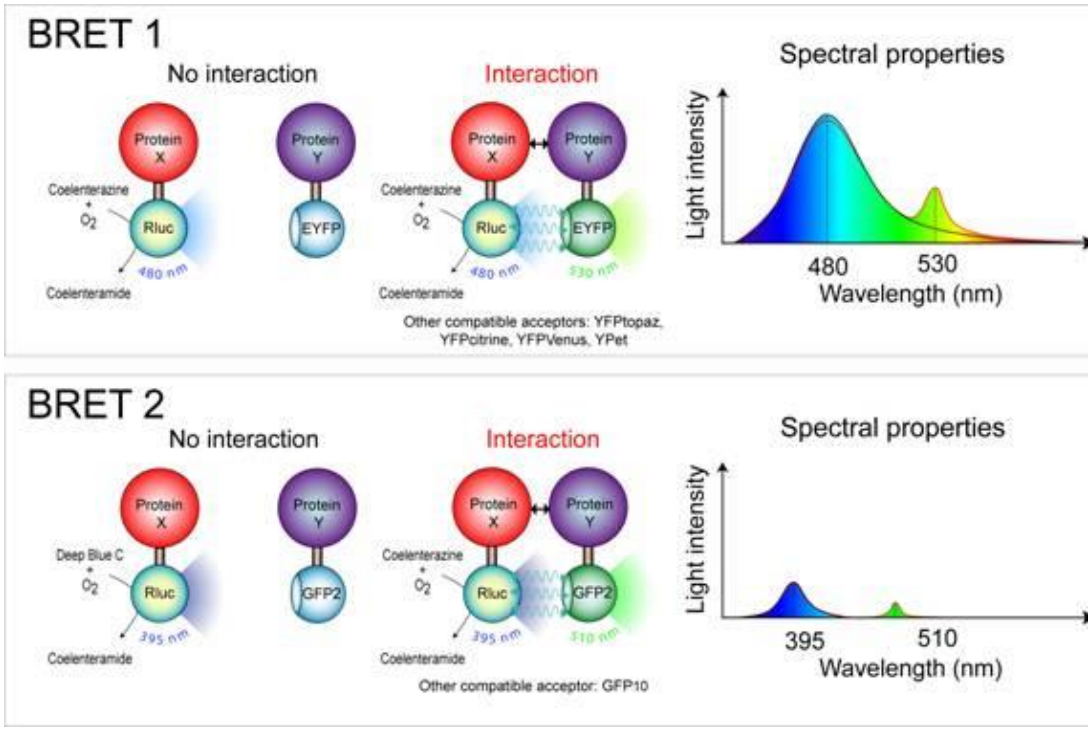

Figure 24. Description of BRET^1 and BRET^2 assays. *Extracted from Institut Cochin.*

Acceptor and donor moieties can be fused to proteins of interest and following expression of the chimeric proteins in cells, production of an energy transfer signal indicates that the donor and acceptor are in proximity, and by extension, the proteins to which they are attached.

In this Thesis, all of the BRET experiments performed are BRET[1] and the two pairs used are Rluc8 and Venus. Rluc8 is a variant of Rluc with enhanced enzymatic activity since it is ~4 times brighter than Rluc and more stable in different environments (Loening et al., 2006). Venus is a variant of the YFP, a genetic mutant of the GFP (green fluorescent protein) and it has the advantage of improved maturation and brightness as well as reduced environmental dependence (Nagai et al., 2002; Rekas et al., 2002).

Recently, several studies have applied BRET for the study of dynamic cellular processes, like the modulation of the interaction of two proteins following pharmacological treatment or the development of biosensors for various signaling pathways. The term biosensor is referred to pharmacologically responsive interactions of GPCRs with other interacting proteins designed to study GPCR signaling pathways. BRET biosensors have been instrumental in advancing our understanding of GPCR signal transduction by providing optical tools to study real-time interactions between receptors, the recruitment of binding partners to receptors, and variations in concentrations of second messengers generated downstream of receptors. Most importantly, BRET studies are conducted in living cells and enable the study of a wide variety of signaling systems to be probed under biologically relevant conditions, with minimal perturbation and in a quantitative manner. GPCR-related BRET biosensors have already established utility as screening platforms in drug discovery process. In the GPCR field, BRET has been used most extensively to examine if GPCRs exist as dimers and/or higher order oligomers. Evidence in favor of the existence of homodimers has been produced for a considerable number of receptors, for example β_2-adrenoceptor (Angers et al., 2000), δ-opioid receptor (McVey et al., 2001), A_{2a} receptor (Canals et al., 2004; Bonaventura et al., 2015); and heterodimers, for example somatostatin $SSTR_{2A}$ and $SSTR_{1B}$ (Rocheville et al., 2000), A_{2a} and D_2 receptors (Canals et al., 2003), A_1 and A_{2A} receptors (Ciruela et al., 2006), A_{2A} and CB_1 receptors (Carriba et al., 2007), D_1 and H_3 histamine receptors and D_2 and H_3 (Ferrada et al., 2008, 2009; Moreno et al., 2014), D_1 and D_3 receptors (Marcellino et al., 2008; Guitart et al., 2014), or corticothropin-releasing factor (CRF) and orexin receptors (Navarro et al., 2015), among others.

In this Thesis, several BRET biosensors have been used to investigate the biology, pharmacology, and signaling of GPCRs in a dynamic fashion. The fundamentals of each assay used appear below.

G-protein activation

This technique allows the monitoring of pharmacological interactions between GPCRs and their cognate G proteins in the presence of receptor ligands by measuring BRET between two G protein subunits. It requires the use of tagged-fused G-protein subunits. The first study to utilize BRET to monitor the activation of heterotrimeric G protein after activation of a GPCR came from Bouvier's lab (Galés et al., 2006). In this study they used probes inserted in multiples sites for BRET donors and acceptors to detect real-time ligand-promoted conformational changes in human receptor-G protein signaling complexes in living cells.

In this Thesis, we wanted to compare the efficiency of the activation of different G protein subtypes (different α subunits) in the presence of receptor agonists. To do that we used five different Gαi subunits fused to Rluc8 as the donor and Gγ subunit fused to Venus as the acceptor and BRET was measured after activation of the receptor. Cells are then transfected with the following constructs: Gαi-Rluc8 (with different subtypes), Gγ2-Venus, Gβ1, and the receptor of choice (Fig 25).

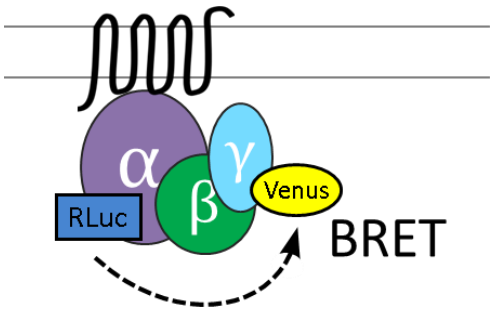

Figure 25. G protein activation. Rluc is fused to the Gα subunit and Venus is fused to the Gγ subunit. BRET decrease between Gα and Gγ is recorded after activation of the receptor by a ligand. *Obtained from Dr. Hideaki Yano.*

G-protein engagement

This assay allows the study of real-time receptor-mediated G protein activation in living cells by using tagged-fused receptor and G protein subunits. In the case of this thesis we used Rluc-fused to the receptor and Venus-fused Gαi subunit. The Rluc is located in the C terminus of the receptor and is fused through a flexible linker. Cells were cotransfected with Gβ1 and Gγ2 to maintain the stoichiometry (Fig 26).

Although drug-induced BRET change reflects receptor activation (Galés et al., 2005), this assay is called G protein engagement to differenciate from G protein activation assay described above involving two G protein subunits. In addition, the use of the expression G protein "engagement" and not "recruitment" is related to a likely precoupled state between receptor and G protein (Galés et al., 2005).

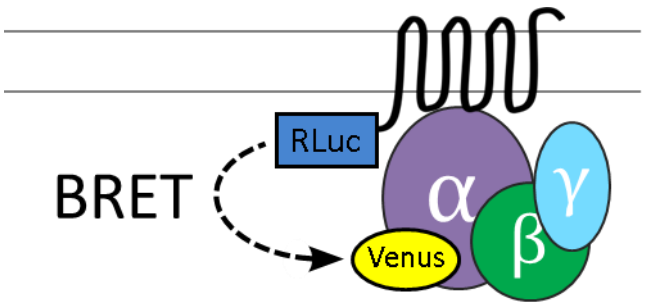

Figure 26. G protein engagement. Rluc is fused to the C terminal of the receptor Venus is fused to the Gα subunit. BRET increase between receptor and Gα is recorded after activation of the receptor by a ligand. *Obtained from Dr. Hideaki Yano.*

β-arrestin recruitment

In this assay, β-arrestin is fused to Venus and receptor is fused to either Rluc or viceversa and drug-induced BRET is measured. In this thesis, we used β-arrestin 2 (Arrestin 3) fused to Venus and Rluc-fused receptor (the same receptor constructs used for G protein engagement BRET) (Fig 27). GRK (G protein-coupled receptor kinases) is cotransfected since it facilitates β-arrestin recruitment (Evron et al., 2012).

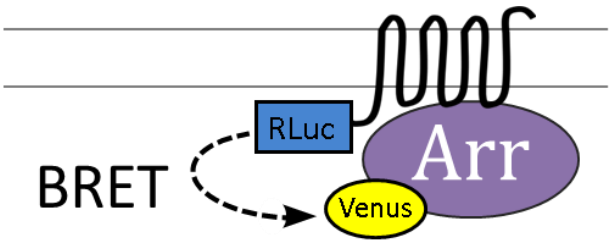

Figure 27. β-arrestin recruitment. Rluc is fused to the C-terminal part of the receptor and Venus is fused to the N-terminal part of arrestin. BRET between Receptor and arrestin is recorded after activation of the receptor by a ligand. *Obtained from Dr. Hideaki Yano.*

The first study to use this method was Angers et al. (2000) which used β-arrestin2-YFP and β_2-Rluc adrenoceptor pairs. Since this seminal study, β-arrestin recruitment has been reported in many manuscripts, where the time course, dose-response, ligand dependency, and effects of receptor homo/hetero-oligomerization on recruitment have been assessed (Pfleger and Eidne, 2003; Pfleger et al., 2007). BRET-based assays for the GPCR/β-arrestin interactions are so robust that they are suitable for high-throughput screening (Bertrand et al., 2002; Hamdan et al., 2005), and they have been developed for many receptors (e.g. chemokine, opiate, dopamine, and prostanoid receptors) (Hamdan et al., 2005; Qiu et al., 2007; Coulon et al., 2008; Klewe et al., 2008; Masri et al., 2008; Leduc et al., 2009).

cAMP accumulation

In order to measure changes of cAMP concentration in real time in living cells, the cAMP biosensor CAMYEL was used. It was developed in 2007 (Jiang et al., 2007) and it consists on a catalytically inactive Epac1 sandwiched between the Rluc and YFP (or Venus). The biosensor detects the conformational changes in Epac that are induced upon its binding to cAMP. The conformational change triggered by an increase in cAMP results in a decrease in BRET presumably due to a change on the relative orientation between the donor and the acceptor (Fig 28). Therefore, a decrease in BRET is observed as an increase of cAMP, typical of Gs-coupled receptors or treatment with forskolin. An increase in BRET is interpreted as a decrease in cAMP such as the one mediated by a Gi-coupled receptor.

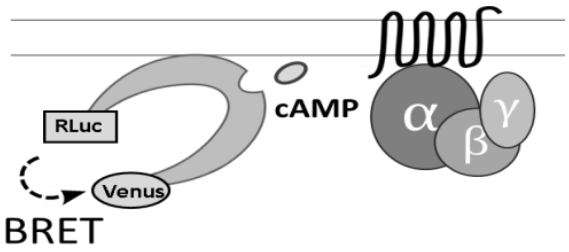

Figure 28. Schematic overview of CAMYEL biosensor. Binding of cAMP to CAMYEL induces a conformational change in the Epac1 resulting in a decrease of energy transfer from the luciferase to the fluorescent protein. *Obtained from Dr. Hideaki Yano.*

CODA-RET

Complemented donor-acceptor resonance energy transfer (CODA-RET) combines bimolecular complementation of either luminescent or fluorescent proteins (Kerppola, 2006ab; Shekhawat and Ghosh, 2011) with RET. It allows the study of a signaling complex comprised specifically of two defined GPCRs (Urizar et al., 2011). This technology is based on quantifying the BRET between a receptor dimer and a subunit of the heterotrimeric G protein. Each receptor is fused to half Rluc on its C-terminal and when the two receptors interact, the two Rluc halves complement producing a functional donor of the BRET. The fluorescent acceptor is fused to one subunit of the heterotrimeric G protein but it can also be extended to the study of arrestins or other interacting partners. In this thesis we used Rluc complementation to control for the oligomerization of receptors and Venus-fused Gα subunit (Fig 29). Drug-induced G protein coupling to the dimer complex (both homomers and heteromers) was studied for dopamine and adrenergic receptors.

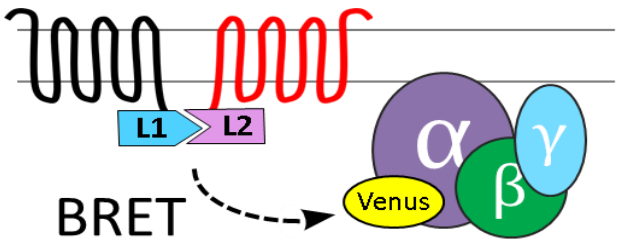

Figure 29. Schematic representation of CODA-RET. Using the complemented receptors as a donor, BRET[1] to an acceptor Venus fused to Gα subunit can be measured. Agonist-stimulated BRET change is

indicative of G protein coupling. L1 and L2 represent the two halves of the Rluc each fused to the C-terminal of one receptor (L1, amino acids 1-229; L2, amino acids 230-311). *Obtained from Dr. Hideaki Yano.*

II. AIMS

II. AIMS

Dopamine is an important neurotransmitter implicated in the regulation of important aspects of brain function including locomotor activity, motivation, memory and addiction. It binds to and activates two families of receptors: D_1-like and D_2-like receptors. In general, dopamine innervation coincides with the distribution of dopaminergic receptors, but this is not always the case in the brain. The cerebral cortex receives dense noradrenergic innervation and even though dopaminergic terminals are more concentrated in the prefrontal cortex (Descarries et al., 1987; Séguéla et al., 1990), the localization of dopamine receptors exceeds that of the dopaminergic terminals. One possible explanation already described for the D_4 receptor (D4R) is the possibility of dopaminergic receptors to be activated by norepinephrine. However, no evidence existed for the other D_2-like receptors. In addition, no clear functional differences have been found between the products of the very prevalent polymorphic variants of the D4R gene. One of these variants in particular, the D4.7R variant, has been repeatedly associated with disinhibition, which is an endophenotype for ADHD and SUD. For these reasons we formulated the following aim:

AIM 1: To study the possible activation of D_2-like receptors (including the three main D4R variants) by norepinephrine and how it compares to dopamine by radiolabeled ligand binding and functional BRET-based assays.

The neurotransmitter norepinephrine has an important role in wakefulness, attention, learning, memory and stress. It binds and activates three families of receptors: α_1 (α_{1A}, α_{1B}, α_{1D}), α_2 (α_{2A}, α_{2B}, α_{2C}) and β (β_1, β_2, β_3). α_{2A}-adrenoceptor (α2AR) is the main subtype in most

brain regions and is highly expressed in the striatum together with α_{2C} (α2CR). The well-appreciated low levels of norepinephrine in the striatum prompted a fundamental question about the function of adrenergic receptors in the striatum. It has been previously postulated that dopamine could provide the endogenous neurotransmitter for striatal α_2-adrenoceptors, although with confounding results, leaving the question unanswered. For these reasons we formulated the following aim:

AIM2: To study the activation of α2AR and α2CR by dopamine and dopaminergic ligands by radiolabeled ligand binding and G protein-subtype activation assay.

It is now well accepted that GPCRs form homo- and heterodimers or high order oligomers at the membrane level. These protein-protein interactions implicate important changes in the pharmacological and functional properties of these receptors, and characterizing them is fundamental to understand their role in the brain as well as for their therapeutic implications. The specific function of D4R in the brain was unknown until recent results from *in vivo* experiments in our lab demonstrated a key role of D4R in the control of corticostriatal transmission. Interestingly, D4.7R provided a gain of function of the receptor, compared to D4.4R. Some evidence suggests that this function may depend on molecular interactions with other receptors since a decreased ability of D4.7R to establish intermolecular and functional interactions with D_2 receptors (D2R) in transfected cells was demonstrated (Borroto-Escuela et al., 2011; González et al., 2012). Similar to D4R, the α_{2A}-adrenoceptor (α2AR) gene contains several polymorphisms that have been associated with ADHD and impulsivity (Roman et al., 2003; Park et al., 2005; Stevenson et al., 2005). In view of the common epidemiological and pharmacological involvement of D4R and α2AR in impulse control and the apparent promiscuity of DA and NE in their ability to activate both Gi/o-coupled receptors, we formulated the third aim of this thesis:

AIM 3: To compare the ability of D4.4R and D4.7R to form functional complexes with D2R and α2AR in transfected cells and activate G protein by different ligands including dopamine and norepinephrine.

III. MATERIAL AND METHODS

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3.1. DNA Constructs and Transfection

For all the receptor constructs, a signal peptide followed by a Flag epitope tag were fused to the N-terminus for enhanced cell surface expression (Guan et al., 1992) and their detection (Guo et al., 2008). Human receptor constructs were used for D2SR, D2LR, D3R, D4.2R, D4.4R, D4.7R (Guitart et al., 2014; Frederick et al., 2015) and α2AR, α2CR (cDNA resource center). For fusion receptor constructs with Renilla luciferase 8 (RLuc8; provided by Dr. S. Gambhir, Stanford University, Stanford, CA), the cDNA encoding full-length RLuc8 was fused in frame to the C terminus of receptors as reported elsewhere (Urizar et al., 2011). The following human G protein constructs were used: Gαi1-mVenus with mVenus inserted at position 91, Gαi1-, Gαi2-, Gαi3-, Gαo1-, or Gαo2-RLuc8 with RLuc8 inserted at position 91, Gαs-RLuc8 with RLuc8 inserted at position 67, Gαq-RLuc8 with RLuc8 inserted at position 97, untagged Gβ1, untagged Gγ2, and Gγ2 fused to full-length mVenus at its N terminus. The Gα-Rluc8 constructs were kindly provided by Dr. Céline Galés (INSERM, Toulouse, France). The cAMP sensor with yellow fluorescence protein (YFP)-Epac-RLuc (CAMYEL) was obtained from the American Type Culture Collection (no. MBA-277; ATCC, Manassas, VA) (Jiang et al., 2007). G protein-coupled receptor kinase 2 (GRK2) and mVenus-fused β arrestin-2 (Vishnivetskiy et al., 2011) constructs were obtained from Dr. J. Javitch (Columbia University, New York, NY). All the constructs were confirmed by sequencing analysis.

A constant amount of plasmid cDNA (15 μg) was transfected into HEK293Tcells using polyethylenimine (Sigma-Aldrich) in a 1 to 2 ratio in 10-cm dishes. Cells were maintained in culture with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum

and kept in an incubator at 37°C and 5% CO_2. The transfected amount and ratio among the receptor and heterotrimeric G proteins were tested for optimized dynamic range in drug-induced BRET. The experiments were performed approximately 48-hours after transfection.

3.2. BRET assays

Variations of BRET were performed to detect receptor ligand-induced events for: 1) Gi/o protein activation, 2) G protein engagement, 3) adenylyl cyclase activity, 4) β arrestin recruitment, and 5) CODA-RET.

Gi/o protein activation assay uses RLuc-fused Gαi/o protein subunit and mVenus-fused Gγ2 protein for resonance energy transfer (RET) pair. Flag-tagged receptor and untagged Gβ1 constructs were cotransfected.

Gi/o protein engagement uses RLuc-fused at the C-terminus of the receptor and mVenus-fused Gi1 protein for resonance energy transfer (RET) pair. Untagged Gβ1 and Gγ2 were cotransfected.

The adenylyl cyclase activity assay uses CAMYEL biosensor construct that contains RLuc and YFP allowing detection of intracellular cAMP change (Jiang et al., 2007). To study Gαi dependent inhibition activity, cells were prestimulated with 1μM or 10 μM forskolin (Sigma-Aldrich). Experiments using Pertussis toxin (PTX) to eliminate the Gαi-mediated Adenylyl cyclase inhibition were performed by treating the cells with 0.1 μg/ml pertussis toxin (Sigma-Aldrich) for 18 h.

The β arrestin recruitment uses RLuc-fused receptor and mVenus-fused β arrestin-2 for the RET pair. GRK2 was cotransfected to assist an enhanced phosphorylation required for the β arrestin recruitment (Evron et al., 2012).

The CODA-RET assay uses receptor fused to Rluc splits for complementation. The L1 split corresponds to residues 1-229 whereas the L2 split corresponds to residues 230-311 and they are fused in frame to the C-terminus of D4.4R, D4.7R, D2SR or α2aR in the pcDNA3.1 vector. To study G-protein activation mediated by dimers, Gαi1-mVenus was used as the acceptor.

The transfected amount and ratio among receptor-L1 and receptor-L2 was optimized by testing various ratios of plasmids.

As reported previously (Urizar et al., 2011), cells were harvested, washed, and resuspended in phosphate-buffered saline). Approximately 200,000 cells/well were distributed in 96-well plates, and 5 µM coelenterazine H (substrate for luciferase) was added to each well. One minute after addition of coelenterazine H, the ligand (dopamine, L-(-)-norepinephrine, clonidine, RO 10-5824, 7-OH-PIPAT, PD 168077, quinpirole, pramipexole, ropinirole, rotigotine) was added to each well. Antagonists were added 10 minutes before the addition of agonist. The fluorescence of the acceptor was quantified (excitation at 500 nm and emission at 540 nm for 1-second recordings) in Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany) to confirm the constant expression levels across experiments.

In parallel, the BRET signal from the same batch of cells was determined as the ratio of the light emitted by mVenus (510–540 nm) over that emitted by RLuc (485 nm). Results are calculated for the BRET change (BRET ratio for the corresponding drug minus BRET ratio in the absence of the drug) 10 minutes (or 20 for β-arrestin assay) after the addition of the ligands. Data and statistical analysis were performed with Prism 5 (GraphPad Software).

3.3. Radioligand Binding Experiments

In this thesis two binding assays were performed; the first was done using HEK cells and the second with brain tissue. They use slightly different methodologies for analyzing the data so I will describe them individually.

3.3.1. HEK 293 cells binding assay

Human embryonic kidney (HEK) 293 cells stably expressing human D2R, D3R, or D4.4R were grown in a 50:50 mix of Dulbecco's modified Eagle's medium and Ham's F-12 culture medium, supplemented with 20 mM HEPES, 2mM L-glutamine, 0.1 mM nonessential amino acids, 1X antibiotic/antimycotic, 10% heat-inactivated fetal bovine serum, and 200 µg/ml hygromycin (Life Technologies, Grand Island, NY) and kept in an incubator at 37°C and 5% CO_2. Upon reaching 80% to 90% confluence, cells were harvested using premixed Earle's Balanced Salt

Solution with 5 µM EDTA (Life Technologies) and centrifuged at 3000 rpm for 10 minutes at 21°C. The supernatant was removed, and the pellet was resuspended in 10 ml hypotonic lysis buffer (5 mM MgCl2, 5 mM Tris, pH 7.4 at 4°C) and centrifuged at 20,000 rpm for 30 minutes at 4°C. The pellet was then resuspended in fresh binding buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4). A Bradford protein assay (BioRad Laboratories, Hercules, CA) was used to determine the protein concentration.

All test compounds were freshly dissolved in 30% dimethylsulfoxide (DMSO) and 70% H_2O to a stock concentration of 1 mM or 100 µM. Each test compound was then diluted into 11 half-log serial dilutions Using 30% DMSO vehicle; final test concentrations ranged from 10 µM to 10 pM in duplicates. Competitive-inhibition experiments were conducted in 96-well plates containing 300 µl fresh binding buffer, 50 µl of diluted test compound, 100 µl of membranes suspension (80 µg/well for D2R, 40 µg/well for D3R and 60 µg/well for D4.4R), and 50 µl of radioligand diluted in binding buffer (7-hydroxy-N,N-di-n-propyl-2-aminotetralin: [^{3}H]7-OH-DPAT; 1.5 nM final concentration for D2R, 0.5 nM final concentration for D3R, 3 nM final concentration for D4.4R; ARC, Saint Louis, MO). Nonspecific binding was determined using 10 µM (+)-butaclamol (Sigma-Aldrich, St. Louis, MO), and total binding was determined with 30% DMSO vehicle.

The reaction was incubated for 90 minutes at room temperature and terminated by filtration through Uni-Filter-96 GF/B (PerkinElmer, Waltham, MA), presoaked in 0.5% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments, Gaithersburg, MD). Filters were washed 3 times with 3 ml of ice-cold binding buffer; 65 µl PerkinElmer MicroScint 20 Scintillation Cocktail was added, and the filters were counted using a PerkinElmer MicroBeta Microplate Counter. The IC_{50} values for each compound were determined from dose–response curves, and the K_i values were calculated using the Cheng-Prusoff equation in GraphPad Prism 5 (GraphPad Software, San Diego, CA). Reported K_i values were determined from at least three independent experiments.

3.3.2. Brain tissue binding assay

Brain tissues were disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for two 5 s-periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4 containing a proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA). Membranes were obtained by centrifugation twice at 37000 rpm for 45 min at 4°C. The pellet was stored at −80°C, washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Membrane protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard. Binding experiments were performed with membrane suspensions at room temperature in 50 mM Tris·HCl buffer, pH 7.4, containing 10 mM $MgCl_2$. For α_2R competition-binding assays, membrane suspensions (0.2 mg of protein/mL) were incubated for 2 h with a constant free concentration of 0.9 nM of the α_2R antagonist [^{3}H]RX821002, and increasing concentrations of each tested ligand: NE, DA, clonidine, 7-OH-PIPAT, quinpirole, RO-105824 and PD-168077. For α_2R saturation-binding assays, membrane suspensions (0.2 mg of protein/mL) were incubated for 3 h at room temperature in 50 mM Tris·HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ with increasing concentrations of the α_2R antagonist [^{3}H]RX821002. Non-specific binding was determined in the presence of 10 µM of the non-radiolabeled antagonist RX821002. In all cases, free and membrane-bound ligands were separated by rapid filtration of 500 µl aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold 50 mM Tris-HCl buffer. The filters were incubated with 10 mL of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 2800 TR scintillation counter (PerkinElmer) with an efficiency of 62%.

Data were analyzed according to the 'two-state dimer model' of Casadó et al. (2007). The model assumes GPCR dimers as a main functional unit and provides a more robust analysis of parameters obtained from saturation and competition experiments with orthosteric ligands, as compared with the commonly used 'two-independent-site model' (Casadó et al., 2007, 2009; Ferré et al., 2014). In saturation experiments with the radioligand, the model analyzes the total number of radioligand binding sites (B_{max}; more specifically it calculates RT, the total number of dimers, where $B_{max} = 2R_T$), the affinity of the radioligand for the first protomer in

the unoccupied dimer (K_{DA1}), the affinity of the radioligand for the second protomer when the first protomer is already occupied by the radioligand (K_{DA2}) and an index of cooperativity of the radioligand (D_{CA}). A positive or negative value of D_{CA} implies either an increase or a decrease in affinity of K_{DA2} versus K_{DA1} and its absolute value provides a measure of the degree of increase or decrease in affinity. In competition experiments the model analyzes the interactions of the radioligand with a competing ligand and it provides the affinity of the competing ligand for the first protomer in the unoccupied dimer (K_{DB1}), the affinity of the competing ligand for the second protomer when the first protomer is already occupied by the competing ligand (K_{DB2}) or the radioligand (K_{DAB}) and an index of cooperativity of the competing ligand (D_{CB}). A positive or negative value of D_{CB} implies either an increase or a decrease in affinity of K_{DB2} versus K_{DB1} and its absolute value provides a measure of the degree of increase or decrease in affinity.

Radioligand competition and saturation curves were analyzed by nonlinear regression using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the binding data to the mechanistic two-state dimer receptor model, as described in detail elsewhere (26, 43). The equation describing the saturation experiment with the radioligand A in non-cooperative conditions ($K_{DA2}/K_{DA1} = 4$) is: $A_{bound} = 2\ A\ R_T / (2\ K_{DA1} + A)$, where A represents de radioligand concentration. To calculate the macroscopic equilibrium dissociation constants from competition experiments, the following general equation must be applied: $A_{bound} = (K_{DA2}\ A + 2A^2 + K_{DA2}\ A\ B / K_{DAB})\ R_T / (K_{DA1}\ K_{DA2} + K_{DA2}\ A + A^2 + K_{DA2}\ A\ B / K_{DAB} + K_{DA1}\ K_{DA2}\ B / K_{DB1} + K_{DA1}\ K_{DA2}\ B^2 / (K_{DB1}\ K_{DB2}))$, where B represents the assayed competing compound concentration. F. For A non-cooperative and non-allosteric modulation between A and B, the equation is simplified due to the fact that $K_{DA2} = 4K_{DA1}$ and $K_{DAB} = 2K_{DB1}$; $A_{bound} = (4K_{DA1}\ A + 2A^2 + 2K_{DA1}\ A\ B / K_{DB1})\ R_T / (4K_{DA1}^2 + 4K_{DA1}\ A + A^2 + 2K_{DA1}\ A\ B / K_{DB1} + 4K_{DA1}^2\ B / K_{DB1} + 4K_{DA1}^2\ B^2 / (K_{DB1}\ K_{DB2}))$. For A and B non-cooperative and non-allosteric modulation between A and B, the equation can be simplified due to the fact that $K_{DA2} = 4K_{DA1}$, $K_{DB2} = 4K_{DB1}$ and $K_{DAB} = 2K_{DB1}$; $A_{bound} = (4K_{DA1}\ A + 2A^2 + 2K_{DA1}\ A\ B / K_{DB1})\ R_T / (4K_{DA1}^2 + 4K_{DA1}\ A + A^2 + 2K_{DA1}\ A\ B / K_{DB1} + 4K_{DA1}^2\ B / K_{DB1} + K_{DA1}^2\ B^2 / K_{DB1}^2)$.

3.4. FACS assay

Fluorescence-activated cell sorting (FACS) was performed to determine the surface expression of each receptor construct, as described elsewhere (Costagliola et al., 1998), with FACS Canto II (BD Biosciences, San Jose, CA). Briefly, a fraction of the same cells used for BRET (∼500,000 cells) were harvested and incubated with anti-Flag monoclonal antibody (Sigma-Aldrich) in FACS buffer (phosphate-buffered saline with 1% bovine serum albumin and 0.1% sodium azide), washed, and incubated with the secondary anti-mouse antibody conjugated with Alexa Fluor 647 (Invitrogen, Carlsbad, CA). Data are normalized to non-specific staining from the non-transfected cells.

IV. RESULTS AND DISCUSSION

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Chapter 1.

Evidence for noncanonical neurotransmitter activation: Norepinephrine as a dopamine D_2-like receptor agonist

As with any other G protein–coupled receptors (GPCR), by classic radioligand binding studies, monoaminergic neurotransmitter receptors have been defined into subclasses of receptors with the name of the neurotransmitter with the highest affinity. As explained previously, dopamine receptors are classified in two families, the Gs/olf-coupled D_1-like receptors, comprising D_1 and D_5 receptors (D1R and D5R) and the Gi/o-coupled D_2-like receptors, comprising D_2, D_3, and D_4 receptors (D2R, D3R, and D4R) (Beaulieu and Gainetdinov, 2011). D_2-like receptors are expressed in brain areas involved in neuropsychiatric disorders and constitute targets for neurologic and antipsychotic medications. Structurally similar, the catecholamine norepinephrine is a metabolite of dopamine that requires dopamine β monooxygenase for the conversion. Therefore, the presence of the enzyme by the cell type distinguishes norepinephrine from dopamine release sites.

In general, norepinephrine and dopamine innervation coincides with the distribution of adrenergic and dopaminergic receptors, respectively, but this afferent-receptor coincidence is not always the case in the brain. Particularly in rodents, the cerebral cortex receives dense and widespread noradrenergic innervation, whereas dopaminergic terminals are more

concentrated in the prefrontal cortex (Descarries et al., 1987; Séguéla et al., 1990). However, dopamine receptors are expressed throughout the cortex, and their localization exceeds that of the dopaminergic terminals (Lidow et al., 1989; Richfield et al., 1989; Goldsmith and Joyce, 1994; Wedzony et al., 2000). It has therefore been suggested that dopamine is a coneurotransmitter in noradrenergic neurons (Devoto and Flore, 2006) or that there is a long-range volume transmission of catecholamines (Rivera et al., 2008; Agnati et al., 2010). In fact, noradrenergic asynaptic varicosities constitute a majority of releasing sites in the cortex (Séguéla et al., 1990). Another mechanism, but specifically invoked for D4R (Lanau et al., 1997; Newman-Tancredi et al., 1997; Rivera et al., 2008; Cummings et al., 2010; Root et al., 2015), is the possibility that dopamine receptors can also be activated by endogenous norepinephrine.

The aim of this chapter which is reflected in the first aim of this thesis, is to investigate how norepinephrine compares with dopamine in ligand binding to effector-specific functional outcomes using bioluminescence resonance energy transfer (BRET) assays for all the D_2-like receptors. A canonical Gi/o noradrenergic receptor, the α_{2A} adrenergic receptor (α2AR), was also analyzed for comparison. This study included the short and long isoforms of D2R (D2SR and D2LR, respectively) and the three most common human polymorphic variants of the D4R gene, with 2-, 4-, or 7-nucleotide tandem repeats in the sequence encoding the third intracellular loop (D4.2R, D4.4R, and D4.7R). Thus, another unresolved issue about D_2-like receptors is the possible functional distinction among different isoform and polymorphic variants. For D2SR and D2LR, distinct presynaptic *versus* postsynaptic localization patterns respectively have been confirmed by various studies (Khan et al., 1998; Centonze et al., 2003; Lindgren et al., 2003), but there is no consensus about their main functional differences at the cellular level. Nevertheless, some studies indicate a differential dependence of D2SR and D2LR on Gαi/o protein subunits for agonist-induced signaling (Gardner et al., 1996; Xu et al., 2002). Although initial studies seemed to indicate that the D4.7R signals with less efficiency than the most common variant D4.4R (Asghari et al., 1995; Jovanovic et al.,1999), more recent studies suggest that their differences might arise from specific protein interactions, including dopamine receptor heteromerization (González et al., 2012b; Ferré et al., 2014).

1.1 Norepinephrine binds to all dopamine D_2-like receptor subtypes with high affinity in stably transfected HEK-293 cells.

Radioligand competition experiments with the D_2-like receptor agonist [^{3}H]7-OH-DPAT (Lévesque et al., 1992) were performed in HEK-293 cells stably transfected with D2SR, D2LR, D3R, or D4.4R, to compare the affinities of dopamine and norepinephrine for the D_2-like receptors. Competition experiments revealed the high affinity (low K_i values) of dopamine for all the D_2-like receptors, with a rank order of D3R>D4.4R≥D2SR=D2LR (Table 1). Consistent with previous reports (Missale et al., 1998), dopamine showed a K_i value for D3R significantly lower (higher affinity) than for D2LR. Norepinephrine also showed high and similar affinity (around 50 nM) for the four D_2-like receptor subtypes, with only 4-fold (D2LR) to 15-fold (D3R) less affinity compared with dopamine (Table 1).

Table 1. Binding affinities of dopamine and norepinephrine for D2LR, D2SR, D3R, and D4.4R

	Dopamine	**Norepinephrine**	**NE/DA (K_i)**
Receptor	K_i (nM)		
D2LR	16 ± 4	62 ± 17	3.9
D2SR	12.5 ± 3.1	75 ± 10	6
D3R	3 ± 1 *	43 ± 15	14.3
D4.4R	6.4 ± 2.0	68 ± 25	10.6

Results are from competitive inhibition experiments of [^{3}H]7-OH-DPAT *versus* dopamine or norepinephrine in HEK-293 cells stably expressing D2LR, D2SR, D3R, or D4.4R. The equilibrium inhibition constant (Ki; nM) is shown for dopamine or norepinephrine. Values are the mean ± S.E.M. of three to six independent experiments. NE/DA, ratio between K_i values of norepinephrine and dopamine for each receptor. *p<0.05: significantly different compared to D2LR (one-way ANOVA, followed by post hoc Newman-Keuls test).

1.2 Norepinephrine signals with similar potency through all dopamine D_2-like receptor subtypes in transiently transfected HEK-293T cells.

D_2-like receptors, by activating Gi/o proteins, inhibit adenylyl cyclase, therefore decreasing intracellular cAMP levels; consequently, the activation of cAMP targets, such as cyclic nucleotide gated channels, exchange protein directly activated by cAMP (Epac) and cAMP-dependent protein kinase or protein kinase A (Antoni, 2012). To find a functional correlate of the results obtained with radioligand binding experiments, we measured D_2-like receptor-mediated inhibition of adenylyl-cyclase by measuring cAMP levels in intact cells transiently transfected with D2LR, D2SR, D3R or the D4R variants D4.2R, D4.4R, or D4.7R, using cAMP sensor with CAMYEL, a kinase-dead Epac I–based cAMP BRET biosensor. The model takes advantage of the conformational changes in Epac that are induced upon its binding to cAMP. The conformational change triggered by cAMP results in a decrease in BRET due to luciferase and YFP changing their relative orientation from each other. A decrease in cAMP levels is therefore observed as an increase in BRET (Fig. 2A) (Jiang et al., 2007). HEK-293T cells are reported to endogenously express β adrenergic receptors (Atwood et al., 2011). Accordingly, norepinephrine in nontransfected cells stimulated Gs-mediated cAMP increase, which could be completely inhibited by the selective β adrenergic blocker propranolol (10 μM; (Fig. 1). Therefore, propranolol was added throughout the cAMP detection experiments to determine transfected D_2-like receptor-mediated responses.

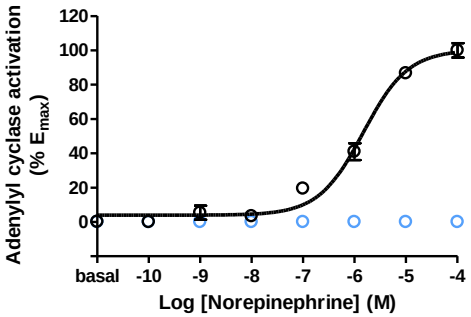

Figure 1. Adenylyl cyclase activation induced by norepinephrine mediated by β adrenergic receptors in non-transfected HEK-293T cells. Dose dependent increase in cAMP without (black) and with the presence of specific β blocker propranolol (blue). Cells were pre-treated with propranolol (10 μM) 10 minutes prior to norepinephrine addition for the antagonist experiment (blue). Cells were treated with coelenterazine H followed by increasing concentrations of norepinephrine. After 10 minutes BRET was measured and basal BRET values were subtracted from BRET values for each norepinephrine concentration. Data represent means ± S.E.M. of 3 to 4 experiments performed in triplicate and are shown as a percentage of the maximal effect.

The assay showed a high sensitivity and provided EC_{50} values for dopamine and norepinephrine that were similar, albeit slightly lower, to the K_i values observed in radioligand binding experiments (Fig. 2, Table 2). The slightly higher potency, particularly of dopamine, in the adenylyl cyclase inhibition assay compared with the radioligand displacement assay may be due to an artifactual ceiling effect set by potentially low cellular expression of the biosensor in a certain population of transfected cells. Depending on the proportion of low-expressing cells, the response of the decrease in cAMP production, read as an increase in BRET, may be seen prematurely as fully efficacious, giving a low estimate of EC_{50}.

Same as the binding experiments, both dopamine and norepinephrine showed low EC_{50} values with D3R, which were significantly different compared with D2LR (Table 2). In comparison with the binding experiments, both for dopamine and norepinephrine the EC_{50} values were moderately higher for D2LR than D2SR, although not statistically different for norepinephrine (Table 2). For dopamine, the EC_{50} values with the three D4R isoforms were as low as with D3R, and no statistically significant differences were obtained between the EC_{50} values for dopamine and norepinephrine in cells transfected with D4.2R, D4.4R, or D4.7R. In terms of efficacy, the E_{max} values for both dopamine and norepinephrine were statistically significantly higher for D2SR and lower for D3R compared with D2L, and no differences were observed among the D4R variants (Table 2).

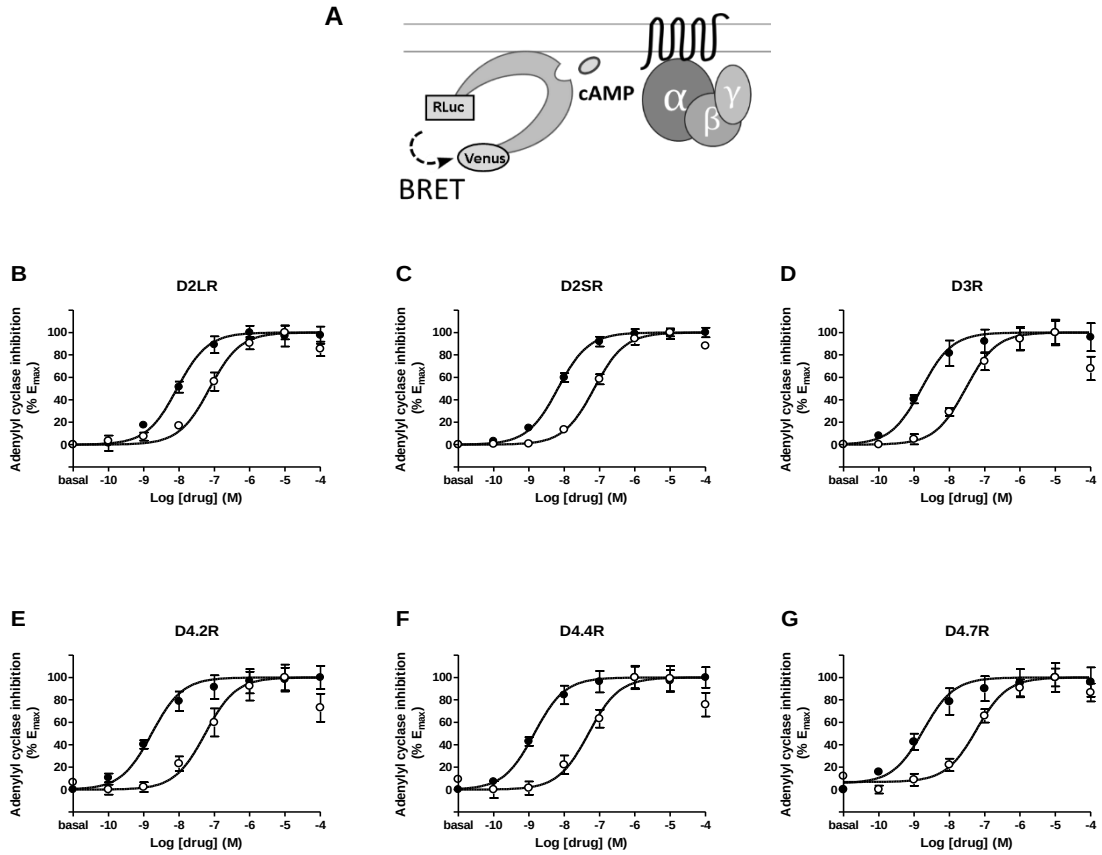

Figure 2. **Adenylyl cyclase inhibition by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R.** (A) Scheme of the CAMYEL sensor configuration where the intramolecular conformational change triggered by cAMP results in a decrease in BRET due to RLuc8 and YFP changing their relative orientation from each other. (B–G) Dose–response experiments of adenylyl cyclase inhibition by dopamine (●) or norepinephrine (○) mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R in HEK-293T cells transiently expressing the CAMYEL sensor and the indicated D_2-like receptor: (B) D2LR, (C) D2SR, (D) D3R, (E) D4.2R, (F) D4.4R, or (G) D4.7R. Cells were treated with forskolin (10 µM) for 10 minutes followed by dopamine or norepinephrine and BRET values obtained by forskolin alone were subtracted from BRET values for each agonist concentration. Data represent the mean ± S.E.M. of 6 to 11 experiments performed in triplicate and are shown as a percentage of the maximal effect (see Table 2 for EC_{50} and E_{max} values and statistical comparisons).

Table 2. Adenylyl cyclase inhibition by dopamine and norepinephrine mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R.

	Dopamine		Norepinephrine		NE/DA (EC_{50})
Receptor	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	
D2LR	9.4 ± 1.1	94 ± 8	88 ± 17	94 ± 6	9.4
D2SR	6.7 ± 0.5 ***	140 ± 7 ***	62 ± 6	149 ± 8 ***	9.3
D3R	1.6 ± 0.2 ***	48 ± 7 ***	19 ± 3 ***	50 ± 5 ***	12
D4.2R	1.8 ± 0.2 ***	74 ± 8	54 ± 8	69 ± 8	30
D4.4R	1.4 ± 0.2 ***	98 ± 9	53 ± 8	79 ± 6	38
D4.7R	1.6 ± 0.3 ***	83 ± 11	53 ± 13	84 ± 6	33

Results are from adenylyl cyclase inhibition-BRET experiments in HEK-293T cells transiently transfected with the CAMYEL sensor and one of the indicated receptors. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration. The EC_{50} and E_{max} values are mean ± S.E.M. of 6 to 11 experiments performed in triplicate. E_{max} values are expressed as 1000 x [BRETmax - BRETbasal]. NE/DA, ratio between EC_{50} values of norepinephrine and dopamine for each receptor. [a]P, 0.001, significantly statistically different compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

1.3 Norepinephrine activates most G proteins with different Gαi/o subunits via D_2-Like receptors at submicromolar concentrations in transiently transfected HEK-293T Cells.

Gαi/o protein subunits are associated with the canonical inhibition of adenylyl cyclase and can be grouped in Gαi, comprising the Gαi1, Gαi2, and Gαi3 subunits, and Gαo, comprising the Gαo1 and Gαo2 splice variants. Studies have suggested the existence of differences in the ability of GPCR to activate G proteins with different Gαi/o subunits, including different D_2-like receptor subtypes (see Discussion and Montmayeur et al., 1993; Liu et al., 1994; Lane et al., 2008), which coincides with different proportions of G protein subunits expressed in different cell types and brain regions (Orford et al., 1991; Jiang et al., 2001). Therefore, using BRET methodology, we compared the ability of dopamine and norepinephrine to activate Gi/o proteins with different Gαi/o subunits via all different D_2-like receptor subtypes, including the

two D2R isoform variants and the three main D4R polymorphic variants. For this purpose, we used luciferase-fused Gαi1, Gαi2, Gαi3, Gαo1, or Gαo2 and Venus-fused γ2 subunit constructs. β1 and γ2 were chosen because of their wide expression in the brain.

As characterized thoroughly in previous reports (Galés et al., 2005, 2006), the ligand-induced G protein conformational change results in negative BRET change, consistent with the opening and distancing between the Gαi and γ2 proteins, which is interpreted as the activation of the heterotrimeric G protein. Taking advantage of a high sequence homology among the five Gαi/o protein subunits, constructs with a luciferase insertion in the same position (i.e., amino acid 91) were used. Luminescence and fluorescence were always measured to control for similar expression levels of transiently transfected G proteins (luciferase-Gαi, β1, and Venus-γ2). Receptor constructs were fused to a Flag epitope at the N terminus, which allowed measurements of cell-surface expression by anti-Flag immunostaining by FACS (Fluorescence-activated cell sorting). To avoid a significant influence by spare receptors, an amount for the receptor cDNA that proportionately increased the surface receptor expression as well as E_{max} was chosen for the study (data not shown). FACS analysis also showed no statistically significant differences in the cell surface expression among the D2R and D4R variants transfected (Fig. 3).

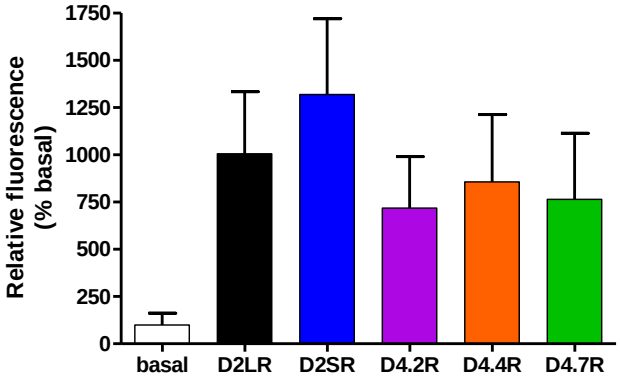

Figure 3. Surface expression of D2LR, D2SR, D4.2R, D4.4R, and D4.7R. Surface expression of the different D2R and D4R variants in cells used for Gi protein activation (transfected with Gαi1 subunit, unfused β_1 and γ_2-mVenus). As described in Materials and Methods, the surface levels of D2R and D4R variants were visualized by anti-flag immunostaining and detected by FACS. Data are normalized to non-specific staining from the non-transfected cells and shown as means ± S.E.M. of 3 to 4 experiments.

The EC_{50} values for both dopamine and norepinephrine were in the submicromolar range for most D_2-like receptors and Gαi/o protein subunits, and they were relatively higher than those obtained in radioligand displacement and adenylyl cyclase inhibition experiments (Fig. 4, Table 3). Gi/o protein coupling specificity was demonstrated by the absence of Gs or Gq protein activation by D_2-like receptors; the absence of dopamine- or norepinephrine-induced Gαi1 protein activation via endogenously expressed receptors was confirmed in cells without transfected D_2-like receptors (Fig. 5). In addition, selective involvement of transfected D_2-like receptors in dopamine and norepinephrine-induced G protein activation (with the Gαi1 subunit) was demonstrated by dose-dependent blockade with the D4R antagonist L745,870 (3-{[4-(4-chlorophenyl)piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine) and the D2R-D3R antagonist raclopride (Fig. 6).

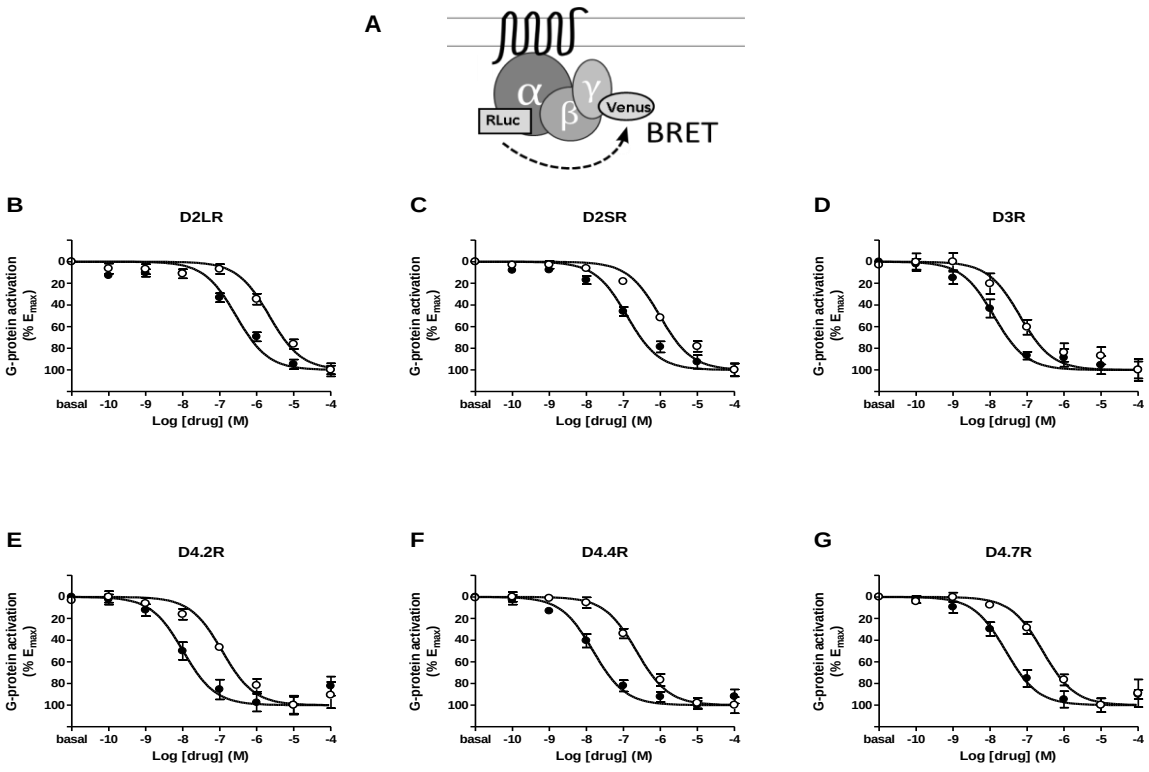

Figure 4. G protein activation by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R. (A) Scheme of the constructs used for G protein activation BRET experiments where RLuc8 is fused to the Gαi1 subunit and mVenus is fused to the γ2 subunit. (B–G) Dose–response experiments of G protein activation by dopamine (●) or norepinephrine (○) mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R in HEK-293T cells transiently expressing the G protein subunits Gαi1-RLuc8, unfused β1 and γ2-mVenus and the indicated D2-like receptor: (B) D2LR, (C) D2SR, (D) D3R, (E) D4.2R, (F) D4.4R, or (G) D4.7R. Cells were treated with coelenterazine H followed by increasing

concentrations of dopamine or norepinephrine. After 10 minutes, BRET between G protein subunits was measured as described in Materials and Methods. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration and are shown as a percentage of the maximal effect. Data represent the mean ± S.E.M. of 11 to 18 experiments performed in triplicate (see Table 3 for EC_{50} and E_{max} values and statistical comparisons).

Table 3. G protein activation by dopamine and norepinephrine mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R involving different Giα/o subtypes.

		Dopamine		**Norepinephrine**		**NE/DA (EC_{50})**
Gα subunit	Receptor	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	
Gαi1	**D2LR**	393 ± 43	19 ± 1	3609 ± 549	17 ± 1	9.2
	D2SR	161 ± 24 [a]	30 ± 1 [a]	987 ± 171 [a]	26 ± 1 [a]	6.1
	D3R	12 ± 3 [a]	15 ± 1	83 ± 33 [a]	15 ± 1	6.9
	D4.2R	18 ± 5 [a]	17 ± 1	116 ± 28 [a]	16 ± 2	6.4
	D4.4R	21 ± 6 [a]	22 ± 1	215 ± 31 [a]	20 ± 1	10.2
	D4.7R	26 ± 5 [a]	25 ± 2 [c]	229 ± 44 [a]	19 ± 1	8.8
Gαi2	**D2LR**	177 ± 46	21 ± 1	1765 ± 391	24 ± 4	10
	D2SR	92 ± 10 *	41 ± 2 [a]	1280 ± 309	39 ± 2 [b]	14
	D3R	NA	NA	NA	NA	
	D4.2R	24 ± 6 [a]	37 ± 3 [a]	340 ± 69 [b]	31 ± 5	14
	D4.4R	19 ± 2 [a]	37 ± 4 [a]	134 ± 30 [b]	35 ± 2 [c]	7
	D4.7R	28 ± 8 [b]	45 ± 3 [a]	285 ± 46 [b]	47 ± 3 [a]	10.2

Gαi3	**D2LR**	132 ± 34	26 ± 2	1198 ± 160	26 ± 3	9.1
	D2SR	52 ± 8 [a]	40 ± 2 [a]	379 ± 103 [a]	40 ± 2 [b]	7.3
	D3R	3.6 ± 0.6 [a]	16 ± 1 [a]	49.4 ± 13 [a]	17 ± 1 [c]	13.7
	D4.2R	21 ± 7 [a]	29 ± 2	316 ± 70 [a]	30 ± 6	15
	D4.4R	12 ± 3 [a]	42 ± 5 [a]	476 ± 116 [a]	34 ± 3	39.7
	D4.7R	15 ± 2 [b]	38 ± 3 [c]	398 ± 102 [a]	39 ± 4 *	26.5
Gαo1	**D2LR**	182 ± 42	35 ± 2	534 ± 67	31 ± 2	2.9
	D2SR	24 ± 4 [c]	36 ± 1	159 ± 26 [c]	36 ± 2	6.6
	D3R	2 ± 0.3 [c]	22 ± 1 [c]	33 ± 11 [c]	21 ± 2	16.5
	D4.2R	197 ± 45	53 ± 3 [b]	408 ± 118	54 ± 4 [a]	2.1
	D4.4R	225 ± 44	57 ± 3 [a]	403 ± 78	54 ± 3 [a]	1.8
	D4.7R	228 ± 38	60 ± 4 [a]	557 ± 114	58 ± 4 [a]	2.4
Gαo2	**D2LR**	227 ± 41	64 ± 1	727 ± 53	55 ± 2	3.2
	D2SR	49 ± 14 [a]	64 ± 2	298 ± 68 [a]	60 ± 3	6.1
	D3R	3 ± 0.4 [a]	33 ± 2 [a]	49 ± 19 [a]	30 ± 3 [a]	16.3
	D4.2R	36 ± 16 [a]	72 ± 6	81 ± 26 [a]	61 ± 5	2.3
	D4.4R	50 ± 14 [a]	69 ± 4	275 ± 55 [a]	63 ± 3	5.5
	D4.7R	128 ± 51 [c]	63 ± 5	122 ± 36 [a]	57 ± 5	0.95

Results were obtained from G protein activation-BRET experiments in HEK-293T cells transiently transfected with different Gαi-RLuc8 subtypes (Gαi1, Gαi2, Gαi3, Gαo1, Gαo2) and γ2-mVenus in cells expressing different D_2-like receptors. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration. The EC_{50} and E_{max} values are the mean ± S.E.M. of 5 to 18 experiments performed in triplicate. E_{max} values are expressed as 1000 x [BRETmax - BRETbasal]. NA, not adjusted. NE/DA, ratio between EC_{50} values of norepinephrine and dopamine. [a]P < 0.001 compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test). [b]P < 0.05 compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test). [c]P< 0.01 compared with D2LR (one-way analysis of variance, followed by post hoc Newman-Keuls test).

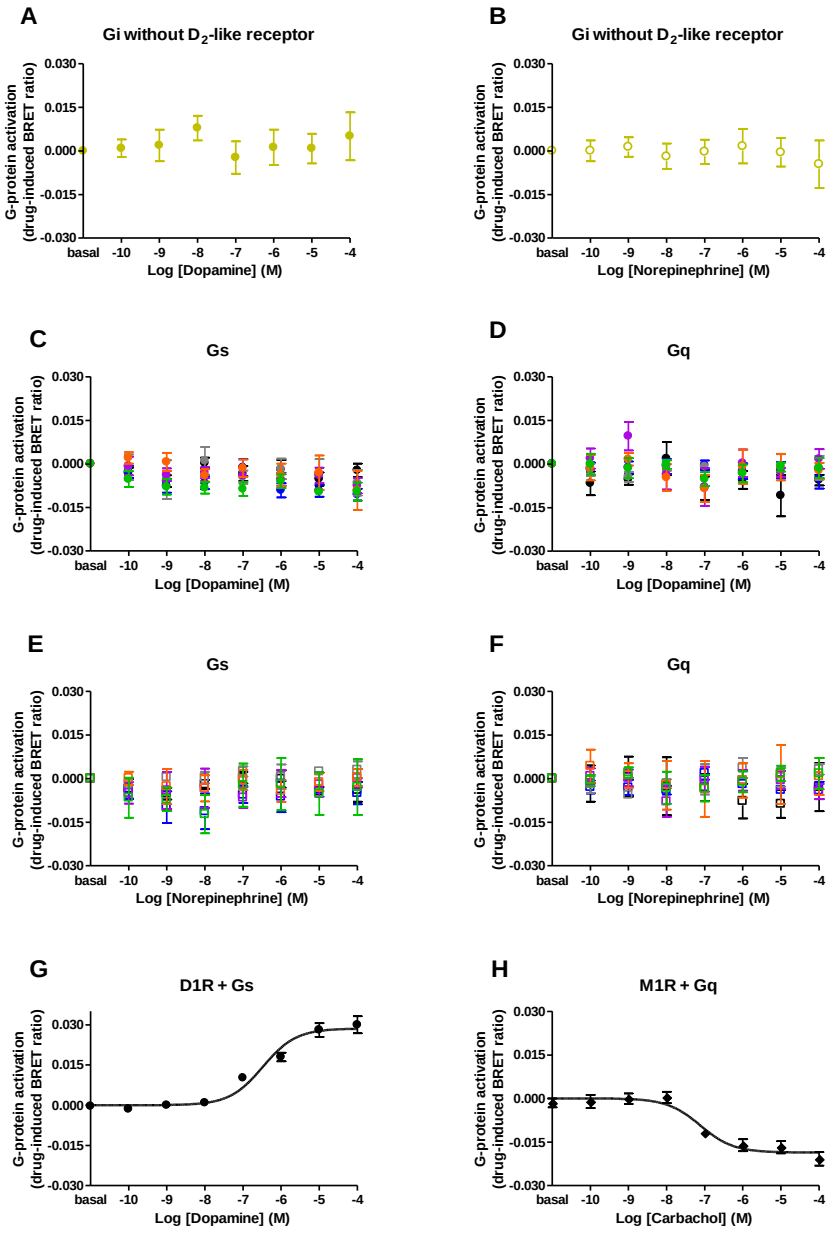

Figure 5. Specificity of dopamine and norepinephrine-mediated activation of Gi by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R. G protein activation-BRET experiments showing the absence of G protein activation by dopamine (filled symbols) or norepinephrine (open symbols) in HEK-293T cells transfected with Gαi1-Rluc subunit without D_2-like receptors (A-B), or transfected with Gαs-Rluc subunit (C-D) or with Gαq-Rluc subunit (E-F) with D2LR (black), D2SR (blue), D3R (gray), D4.2R (purple), D4.4R (orange) or D4.7R (green) and with and the indicated D_2-like receptor. Cells were also transfected with β_1 and γ_2-mVenus. To show the validity of the Gs and Gq biosensors, cells were transfected with Gαs-Rluc subunit and dopamine D_1 receptor (D1R) (G) or Gαq-Rluc subunit with muscarinic M_1 (M1R) receptor (H) together with unfused β_1 and γ_2-mVenus. Cells were treated with coelenterazine H followed by increasing concentrations of dopamine (A, C, D, G), norepinephrine (B, E, F) or carbachol (H). After 10 minutes BRET was measured as described in Materials and Methods. Basal BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were fit by non-linear regression to a sigmoidal

dose- response relationship against the agonist concentration (G, H). Data are means ± S.E.M. of 3 to 4 experiments performed in triplicate.

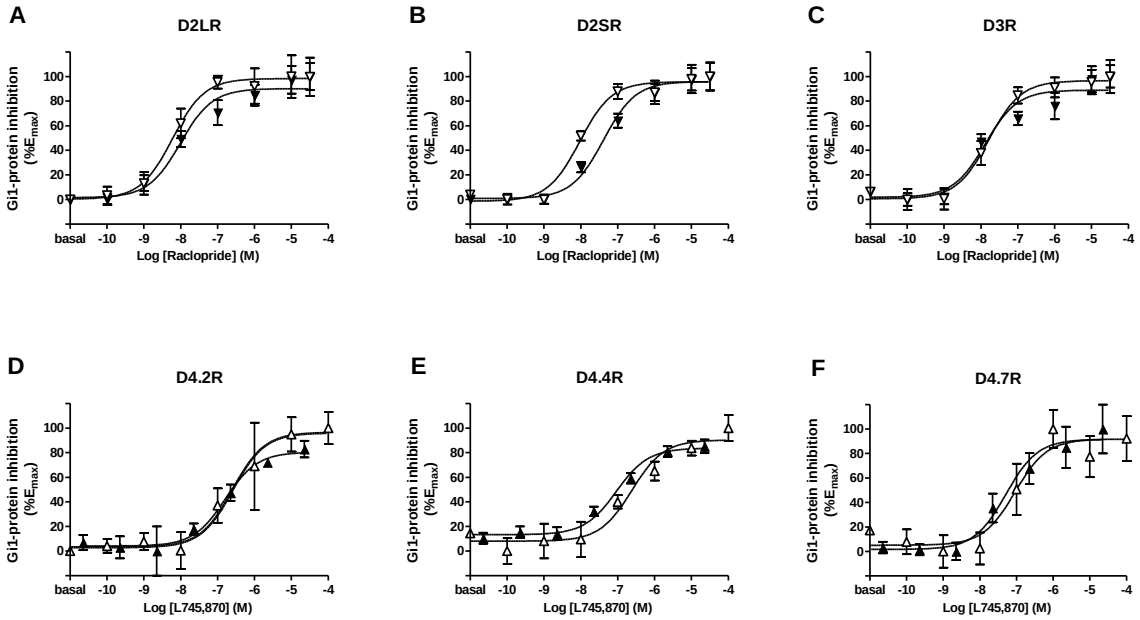

Figure 6. Inhibition of Gi protein activation by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R. Dose-dependent inhibition by selective D2R-D3R antagonist raclopride and D4R antagonist L745,870 of Gi protein activation in HEK-293T cells transfected with Gi protein (Gαi1 subunit, unfused β_1 and γ_2-mVenus) induced by dopamine (filled triangles) or norepinephrine (open triangles) mediated by (A) D2LR, (B) D2SR, (C) D3R, (D) D4.2R, (E) D4.4R or (F) D4.7R. Agonists (10 μM for D2LR, D2SR and D3R and 1 μM for D4.2R, D4.4R and D4.7R) were added 10 minutes after antagonist incubation. BRET was measured as described in Materials and Methods. BRET values for the agonist alone were subtracted from the BRET values for each antagonist concentration. Data were fit by non-linear regression to a sigmoidal dose-response relationship against the antagonist concentration and is shown as a percentage of the maximal inhibitory effect. Data are means ± S.E.M. of 3 to 9 experiments performed in triplicate.

Dopamine was more potent than norepinephrine for most D_2-like receptors and Gαi/o protein subunits, but significant Gαi/o protein subunit-dependent differences were observed (Table 3). For the two D2R and the three D4R variants, dopamine was around 10 times more potent than norepinephrine when coupling to Gαi1, Gαi2, or Gαi3, but only around 2 times more potent when coupling to Gαo1 or Gαo2 (Table 3). Dopamine and norepinephrine showed higher potencies for the D4R than for the D2R variants when coupled to Gαi1 and Gαi2 proteins but similar potencies when coupled to Gαo1 or Gαo2. When coupling to Gαi3,

dopamine was more potent for D4R than for D2R, but norepinephrine showed similar potencies. Dopamine and norepinephrine showed higher potency for D3R than for the other D_2-like receptors with all Gαi/o protein subunits with the exception of Gαi2, which seemed to be completely insensitive to dopamine or norepinephrine-induced D3R activation. No statistically significant differences between D4R variants were observed. On the other hand, a significantly higher potency of dopamine and norepinephrine could be observed at D2SR compared with D2LR with all Gαi/o protein subunits but Gαi2. The same as for the adenylyl cyclase signaling experiments, for most Gαi/o protein subunits the E_{max} values for both dopamine and norepinephrine were significantly higher for D2SR and lower for D3R compared with D2L, and no major differences were observed among the D4R variants (Table 3).

1.4. Norepinephrine promotes β arrestin-2 recruitment to D_2-Like receptors in transiently transfected HEK-293T cells.

Receptor activation also leads to β arrestin-mediated receptor recruitment and its own signaling events (Shenoy and Lefkowitz, 2011), which are major effector-triggered events besides the G protein-mediated signal transduction. Hence, dopamine and norepinephrine-induced β arrestin recruitment across all the D_2-like receptors were also analyzed. RLuc8-fused receptor constructs (Urizar et al., 2011) were transfected with Venus-fused β arrestin-2 and GRK2, whose phosphorylation precedes and assists β arrestin recruitment (Evron et al., 2012). Although right-shifted compared with the G protein-related assays, the EC_{50} values were in agreement with previous reports (Namkung et al., 2009; Clayton et al., 2014). Notably, consistent with the radioligand-binding and G protein–related experiments, the rank order of potency was D3R > D4R > D2R for both dopamine and norepinephrine, and no variant differences were observed (Fig. 7, Table 4). The E_{max} values for both dopamine and norepinephrine were very similar across all dopamine receptor subtypes (Table 4).

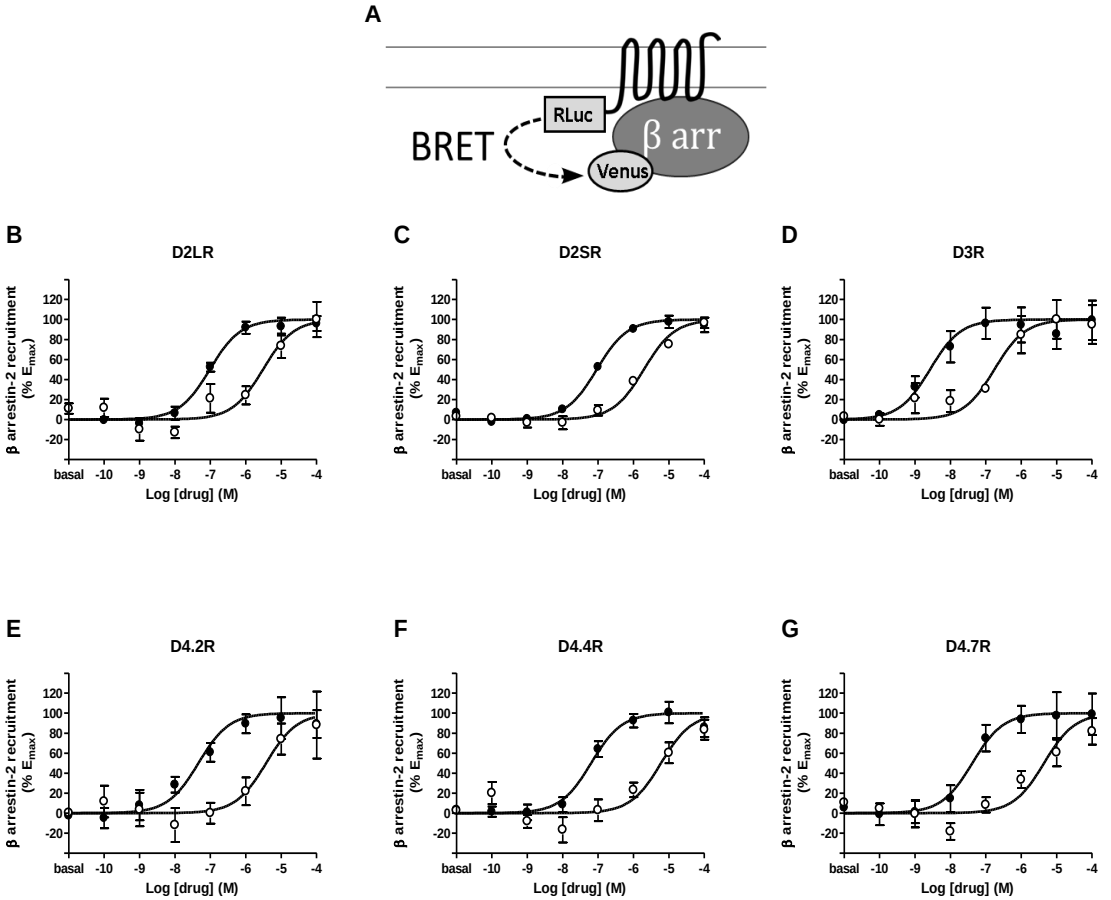

Figure 7. β arrestin-2 recruitment by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R. (A) Scheme of the constructs used for β arrestin-2 recruitment-BRET experiments with different D_2-like receptors fused to RLuc8 and β arrestin-2 fused to mVenus. (B–G) Dose–response experiments of β arrestin-2 recruitment by dopamine (●) or norepinephrine (○) mediated by (B) D2LR, (C) D2SR, (D) D3R, (E) D4.2R, (F) D4.4R, or (G) D4.7R in HEK-293T cells transiently expressing the indicated D_2-like receptor fused to RLuc8, β arrestin-2-mVenus, and GRK2. Cells were treated with coelenterazine H followed by increasing concentrations of dopamine or norepinephrine. After 20 minutes, BRET was measured as described in Materials and Methods. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration and are shown as a percentage of the maximal effect. Data are the mean ± S.E.M. of 5 to 10 experiments performed in triplicate (see Table 4 for EC_{50} and E_{max} values and statistical comparisons).

Table 4. β arrestin-2 recruitment induced by dopamine and norepinephrine mediated by D2LR, D2SR, D3R, D4.2R, D4.4R, and D4.7R

	Dopamine		Norepinephrine		NE/DA (EC_{50})
Receptor	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	
D2LR	96 ± 16	11 ± 1	4800 ± 1720	11 ± 2	50
D2SR	105 ± 21	18 ± 2 **	2880 ± 1150	17 ± 2	27.4
D3R	3 ± 1***	15 ± 2 *	32 ± 5 *	13 ± 2	10.7
D4.2R	38 ± 13 *	9 ± 1	3750 ± 1620	10 ± 2	100
D4.4R	66 ± 13	8 ± 1	2830 ± 666	7 ± 1	43
D4.7R	44 ± 12 *	9 ± 1	1840 ± 889	8 ± 2	41.8

Results were obtained from β arrestin-2 recruitment-BRET experiments in HEK-293T cells transiently transfected with different D_2-like receptors fused to RLuc8 and β arrestin-2 fused to mVenus. Data of individual experiments were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration. The EC_{50} and E_{max} values are the mean ± S.E.M. of 5 to 15 experiments performed in triplicate. E_{max} values are expressed as 1000 x [BRETmax - BRETbasal]. NE/DA, ratio between EC_{50} values of norepinephrine and dopamine for each receptor. *, ** or ***: $p<0.05$, 0.01 or 0.001, respectively, compared to D2LR (one-way ANOVA, followed by post hoc Newman-Keuls test).

1.5 Norepinephrine activates G protein, inhibits Adenylyl Cyclase, and promotes β arrestin-2 recruitment via α2AR at concentrations 20-fold lower than D_2-Like receptors.

The α2AR is a canonical Gi-coupled receptor for norepinephrine that is highly expressed in the prefrontal cortex (Wang et al., 1996) where D2R and D4R also are expressed (Missale et al., 1998). The EC_{50} values of norepinephrine for the G protein activation and adenylyl cyclase inhibition assays were both at the nanomolar range and just about 20-fold lower compared with those of D3R and D4R (Fig. 8, Table 5). Similarly, the potency of norepinephrine for β arrestin-2 recruitment with α2AR was 4-fold higher than with D_2-like receptors, in agreement with their potency separation on G protein activation (Fig. 8, Table 5).

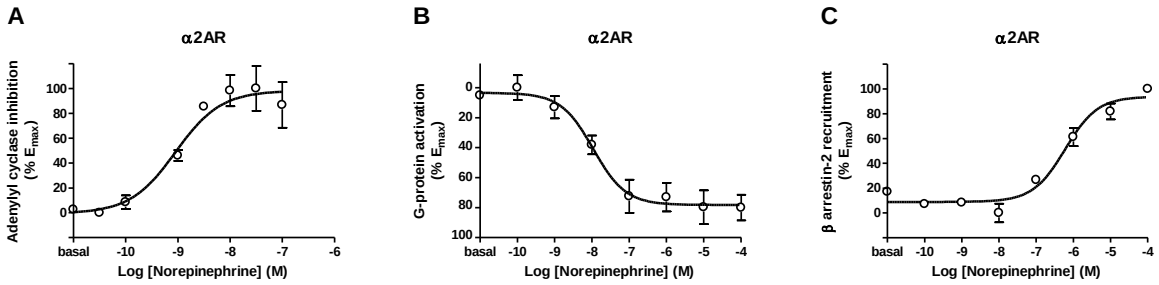

Figure 8. Adenylyl cyclase inhibition, Gαi1 protein activation and β arrestin-2 recruitment by α2AR. (A–C) Dose–response experiments of norepinephrine on α2AR function. (A) Adenylyl cyclase inhibition assay using CAMYEL biosensor. (B) G protein activation assay using Gαi1 and γ2 subunits. (C) β Arrestin-2 recruitment assay using β arrestin-2-mVenus and GRK2. Conditions for each assay are identical to the ones described in Figures 1–3. Basal BRET values for forskolin alone (A) or in the absence of ligands (B, C) were subtracted from the BRET values for each agonist concentration. Data were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration and are shown as a percentage of the maximal effect. Data are the mean ± S.E.M. of three to four experiments performed in triplicate (see Table 5 for EC_{50} and E_{max} values and statistical comparisons).

Table 5. Adenylyl cyclase inhibition, G-protein activation and β arrestin-2 recruitment induced by norepinephrine mediated by α2AR.

	Norepinephrine	
BRET assay	EC_{50} (nM)	E_{max}
Adenylyl cyclase inhibition	1 ± 0.2	76 ± 10
G-protein activation (Gi1)	12 ± 2	24 ± 2
β arrestin-2 recruitment	687 ± 247	15 ± 1

Data of individual experiments were fit by nonlinear regression to a sigmoidal dose–response relationship against the agonist concentration. The EC_{50} and E_{max} values are the mean ± S.E.M. of 3 to 4 experiments performed in triplicate. E_{max} values are expressed as 1000 x [BRETmax - BRETbasal].

1.6 Discussion

From all D_2-like receptor subtypes, to our knowledge, only D4R has been suggested to be a promiscuous" receptor that can be activated by both dopamine and norepinephrine and function as an adrenergic receptor in the brain (Lanau et al., 1997; Newman-Tancredi et al., 1997; Czermak et al., 2006; Cummings et al., 2010; Root et al., 2015). Our present study indicates that all D_2-like receptor subtypes can bind with high affinity and be activated by norepinephrine and that their potencies, particularly for D3R and D4R, are only ∼20-fold lower than for α2AR activation. Application of BRET assays that allow measuring activation of specific G protein subtypes has provided evidence for a significant differential dependence on Gαi/o protein subunits on the ability of both catecholamines to activate D_2-like receptors. Relative potencies obtained with the four assays used in this study were largely consistent, verifying the validity of potencies for the norepinephrine-mediated G protein activation. Furthermore, the study does not support differences in the ability of dopamine or norepinephrine to activate the three main D4R polymorphic variants but does support differences in the two D2R isoform variants for their ability to couple with specific Gαi/o protein subunits.

Several studies have reported that norepinephrine can bind with high affinity and activate D4R with lower potency compared with dopamine in mammalian transfected cells. The difference in agonist potency varied significantly depending on the study, ranging from 2 to more than 50 times (Lanau et al., 1997; Newman-Tancredi et al., 1997; Czermak et al., 2006; Cummings et al., 2010). Part of this variability could certainly be related to methodologic differences in radioligand binding, G protein activation, or effector activation assays used. But our study highlights the importance of the Gαi/o protein subunit involved in D4R activation. Thus, with the three main human D4R polymorphic variants—D4.2R, D4.4R, and D4.7R—dopamine was approximately 10 times more potent than norepinephrine at activating G proteins containing Gαi1, Gαi2, or Gαi3 subunits; it was only twice as potent at activating G proteins containing Gαo1 or Gαo2 subunits. No statistically significant differences with the potencies of dopamine or norepinephrine could be observed among the three D4R polymorphic variants coupled to any of the Gi/o protein subtypes. This extends previous results showing no differences in the ability of dopamine to activate the three different D4R

variants reconstituted with purified G proteins containing Gαi1, Gαi2, or Gαi3 subunits (Kazmi et al., 2000), which questions the studies that have suggested D4R variant-dependent differences in the potency of dopamine or norepinephrine to activate G protein ([^{35}S]GTPγS binding; Czermak et al., 2006) or to inhibit adenylyl cyclase (Asghari et al., 1995). As expected, we did not find any difference in the ability of dopamine or norepinephrine to inhibit adenylyl cyclase or to promote β arrestin recruitment between cells transfected with D4.2R, D4.4R, or D4.7R.

Previous studies reported a low potency of norepinephrine to bind and activate D2R (Lanau et al., 1997; Newman-Tancredi et al., 1997); however, after analyzing agonist binding, G protein activation, adenylyl cyclase inhibition, and β arrestin recruitment, we provide clear evidence for a similar catecholamine "promiscuity" of D2R and D3R as for D4R. The same as for D4R, Gαi/o protein subunit-dependent differences were demonstrated in the potencies of dopamine and norepinephrine for D2SR, D2LR, and D3R. The most striking finding was the selective inability of dopamine or norepinephrine to activate G proteins containing the Gαi2 subunit in D3R-transfected cells, which agrees with previous results from experiments with receptor-G protein fusion proteins and pertussis toxin resistant mutants of G proteins containing different Gαi or Gαo subunits (Lane et al., 2008). Lane and colleagues reported a very efficient agonist-induced coupling of D3R to Gαo1 and an inability of D3R to couple not only to Gαi2 but also to Gαi1 or Gαi3. In our study we were also able to confirm a high potency of dopamine (and norepinephrine) to induce D3R-mediated coupling to Gαo1 and Gαo2 as well as to Gαi1 and Gαi3. Different methodologies should explain the discrepancies between both studies, but in support of our results not only dopamine but also norepinephrine were more potent at D3R than at D2LR or D2SR to induce Gαi1 and Giα3 activation. Furthermore, our results with G protein activation are congruent with our signaling experiments because HEK-293 cells have been reported to express predominantly Gαi1, Gαi2, and Gαi3 and statistically nonsignificant levels of Gαo1 and Gαo2 (Atwood et al., 2011). Thus, both dopamine and norepinephrine were more potent at D3R than at D2LR or D2SR to inhibit adenylyl cyclase or recruit β arrestin. Even though G proteins of the Gαs-Gαolf family do show contrasting brain expression pattern (Hervé 2011), to our knowledge no clear region-specific pattern of mRNA expression for Gαi/o protein subtypes has been reported. Detailed characterization of expression patterns for Gαi/o protein subtypes would be central to

determine their stoichiometric relationship with the different D_2-like receptors and thus the possible contribution of dopamine and norepinephrine in their activation.

Although still a matter of intense debate, as for the human D4R polymorphic variants there is no consensus about the main functional differences between the two D2R isoform variants (Gardner et al., 1996; Xu et al., 2002). Nevertheless, two research groups have independently reported a differential dependence of D2LR and D2SR on G protein subtypes for agonist-induced signaling. Specifically, D2LR has been reported to interact efficiently with the Gαi2 subunit and poorly with the Gαi1 and Gαi3 subunits, while D2SR does not discriminate between these three G protein subunits (Montmayeur et al., 1993; Liu et al., 1994; Guiramand et al., 1995). It has also been suggested that the differential G protein subunit dependence is also effector dependent.

Thus, G proteins containing Gαi1, Gαi2, or Gαi3 subunits would selectively couple and inhibit adenylyl cyclase whereas G proteins containing Gαo subunits would couple and close calcium channels (Liu et al., 1994). In our study, we did not find an absolute selective ability of D2LR to activate Gαi2-containing G protein, but a relative Gαi2 preference compared with D2SR and a higher potency of dopamine and norepinephrine at D2SR compared with D2LR were observed with most Gαi/o subunits. The concomitant expression of Gαi1, Gαi2, and Gαi3 subunits in HEK-293 could explain the higher potency of dopamine at D2SR to inhibit adenylyl cyclase, compared with D2LR (Montmayeur et al., 1993).

The α2AR is functionally relevant to the D_2-like receptors among the canonical norepinephrine adrenergic receptors because it is coupled to Gi/o proteins and highly expressed in the prefrontal cortex (Wise et al., 1997) where D_2-like receptors are also expressed, especially D2R and D4R (Missale et al., 1998). The α2AR is considered a high-affinity receptor for norepinephrine with a reported K_i value of as low as 15 nM in transfected cells (O'Rourke et al., 1994). This is only 3- to 5-fold higher affinity compared with the K_i values of norepinephrine for D_2-like receptor reported herein. Our functional readouts of Gi activation, adenylyl cyclase inhibition, and β arrestin recruitment showed greater but still relatively small separation between the potencies of norepinephrine and dopamine for α2AR

and D_2-like receptors (20-fold), verifying D_2-like receptors as potential signal transducers for norepinephrine.

With our findings on relative potencies, the previously reported so-called cross reactivity phenomenon between dopamine and norepinephrine (Guiard et al., 2008; González et al., 2012a; Lei, 2014) can now be better explained at the receptor-effector levels and considered as likely biologically occurring events. Furthermore, dopamine and norepinephrine concentrations in prefrontal cortex are comparable (Koob et al., 1975; Blank et al., 1979; Li et al., 1998), suggesting that actions of norepinephrine at cortical D_2-like receptors are likely functionally significant.

In conclusion, norepinephrine is a potent agonist for D_2-like receptors and, like dopamine, follows a general rank order of potency of D3R > D4R ≥ D2SR ≥ D2L in most functional assays. If D4R functions as an adrenergic receptor in some brain regions (Root et al., 2015), the other D_2-like receptors—which in fact can be expressed with higher density in some of those areas—should also be considered as functional adrenergic receptors. Some intriguing differences in G protein activation have emerged, which depend on the Gαi and Gαo subunits involved. Although the framework of similarity among different effectors as well as between the two catecholamines may serve as a foundation for future studies on modulatory effects such as protein–protein interactions, the dissimilarities may be potentially pursued for further pharmacologic investigations.

The results of this chapter are published with the title: **Evidence for Noncanonical Neurotransmitter Activation: Norepinephrine as a Dopamine D_2-Like Receptor Agonist.** Sánchez-Soto M, Bonifazi A, Cai NS, Ellenberger MP, Newman AH, Ferré S, Yano H. *Mol Pharmacol.* 2016 Apr;89(4):457-66. doi: 10.1124/mol.115.101

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Chapter 2.

α_{2A}- and α_{2C}-Adrenoceptors as Targets for Dopamine and Dopamine Receptor Ligands

The neurotransmitter norepinephrine (NE) binds and activates three subfamilies of adrenoceptors: α1-adrenoceptors, subdivided into α_{1A}, α_{1B} and α_{1D}; α_2-adrenoceptors, subdivided into α_{2A}, α_{2B} and α_{2C}; and β-adrenoceptors, subdivided into β_1, β_2 and β_3 (Alexander et al., 2011). Classically, α_1-, α_2- and β-adrenoceptors couple to Gq/11, Gi/o and Gs, respectively (Alexander et al., 2011; MacDonald et al., 1997). As described in the introduction, in mammalian species, α_{2A} -adrenoceptor (α2AR) is the main subtype in most brain regions whereas α_{2B} subtype has a limited distribution and is mostly expressed in the thalamus (Nicholas et al., 1993; Scheinin et al., 1994). The α_{2C}-adrenoceptor subtype (α2CR) is found with particularly high density in the striatum (Fagerholm et al., 2008; Lehto et al., 2015) with a moderately lower density than α2AR (Ordway et al., 1993; Uhlen et al., 1997). The high density of striatal α2AR and α2CR prompted a fundamental question in view of the well-known paucity of striatal noradrenergic terminals (Lindvall et al., 1975; Swanson and Hartman, 1975, Aston-Jones, 2004) and the concomitant low extracellular levels of striatal NE (Gobert et al., 2004). Yet a series of studies indicate that both types of receptors are fully functional in the striatum, where they seem to be localized mostly postsynaptically, preferentially in GABAergic striatal efferent neurons (Holmberg et al., 1999; Hara et al., 2010). There is also evidence for α2AR playing a role as autoreceptors localized in the sparse striatal

noradrenergic terminals (Ihalainen and Tanila, 2004). It was postulated by Zhang et al. (1999) (Zhang et al., 1999) that dopamine (DA) could provide the endogenous neurotransmitter for striatal α_2 adrenoceptors. In transfected mammalian cells, using radioligand binding experiments, they found evidence for just a small preferential affinity of NE *versus* DA at both a α2AR and α2CR adrenoceptors. Similar results were more recently obtained from radioligand binding studies using transfected mammalian and insect cell lines (Alachkar et al., 2010) and with autoradiographic experiments in bird and rat brain with a nonselective α_2 adrenoceptor ligand (Cornil and Ball, 2008). However, Zhang et al. (1999) (Zhang et al., 1999) reported a much lower potency of DA than NE at the level of α_2 adrenoceptor-mediated signaling (modulation of forskolin-induced adenylyl cyclase activation), therefore leaving unanswered the question of DA as a possible effective ligand for striatal α2AR and α2CR.

After showing in Chapter 1 of the Results section that norepinephrine has a significant role as a dopamine D_2-like receptor agonist as well as observing the fact that the α_2 adrenergic receptor could be activated by dopamine, we decided to extend our study and characterize the effect of dopamine on Gi-coupled adrenergic receptors. So in the present study, we revisited the possible dopaminergic function of α2AR and α2CR using the same methodology used in the first chapter of the results, activation of all Gi/o-coupled DA D_2-like receptors by NE. Thus, the same as for NE, a mismatch between dopaminergic innervation and the density of DA receptors can be found in several brain areas. DA receptors are for instance expressed throughout the cortex, but their localization exceeds that of the dopaminergic terminals (Lidow et al., 1989; Richfield et al., 1989; Goldsmith and Joyce, 1994; Wedzony et al., 2000). Our methodology consists on sensitive BRET-based techniques that allow detection of ligand-dependent interactions between specific receptors and specific G proteins (G protein activation) or receptor-induced activation of effectors (adenylyl cyclase activity) in living cells. This procedure provided clear evidence for a Gi/o protein subtype dependent functional selectivity of the endogenous neurotransmitters DA and NE, illustrating the ability of Gi/o protein subtypes to determine the potency of both catecholamines to activate different D_2-like receptor subtypes. In addition to studying the effect of DA and several DA receptor ligands on G protein activation in mammalian cells transfected with α2AR or α2CR and the different a subunits of Gi/o protein subtypes, we also analyzed their ability to bind to α_2 adrenoceptors in cortical tissue, which predominantly expresses α2AR, and striatal tissue, which expresses

both α2AR and α2CR adrenoceptors. The results provide conclusive evidence for α2AR and α2CR being both NE and DA receptors and common targets for DA D_2-like receptor ligands.

2.1 α2AR and α2CR-mediated G protein activation by DA and synthetic DA receptor ligands.

The G protein activation BRET assay (see Material and Methods) was used to determine the potency and efficacy of DA and several D2-like receptor ligands to activate α adrenoceptors in HEK-293T cells transfected with α2A or α2C adrenoceptor (α2AR or α2CR, respectively) and one of the five different RLuc-fused Gαi/o subunits (Gαi1, Gαi2, Gαi3, Gαo1 and Gαo2) with Venus-fused Gγ2 protein as BRET acceptor pair. A concentration-response of the ligand-induced change in BRET values allows to determine the potency as well as the relative efficacy (to NE) at α2AR and α2CR-mediated G protein activation. Apart from NE and DA and the non-selective α adrenoceptor agonist clonidine, the following D_2-like receptor ligands were analyzed: the non-selective D2R-D3R-D4R agonist quinpirole, the selective D3R agonist 7-OH-PIPAT and the selective D4R agonists RO-105824 and PD-168077. At both α2AR and α2CR, DA showed high potency and efficacy as compared with NE (Figs. 1 and 2 and Tables 1 and 2). DA was always less potent, but its relative potency to NE depended on the α-adrenoceptor and on the Gαi/o subtype (see Table 1). NE was more potent at α2AR than at α2CR, except for Gαi2 and Gαi3. On the other hand, DA had similar potencies at both adrenoceptors, except for Gαi2 and Gαo1. Therefore, the potencies of DA at activating α2CR were very close to those of NE and they varied from less than two-fold lower, for Gαi1, to about ten-fold lower, for Gαi2 (Table 1). On the other hand, the potencies of DA as compared to NE at activating α2AR varied from about fifteen fold lower, for Gαi1 and Gαo2, to about thirty fold lower, for Gαi3 and Gαo1 (Table 1). In summary, using the G protein activation BRET assay, DA showed high potency at activating both α2AR and α2CR, which became particularly significant when taking into account the very similar or even lower potency of DA for D_2-like receptors using the same BRET construct configurations (Sánchez-Soto et al., 2016; Chapter 1 of the Results, and Discussion).

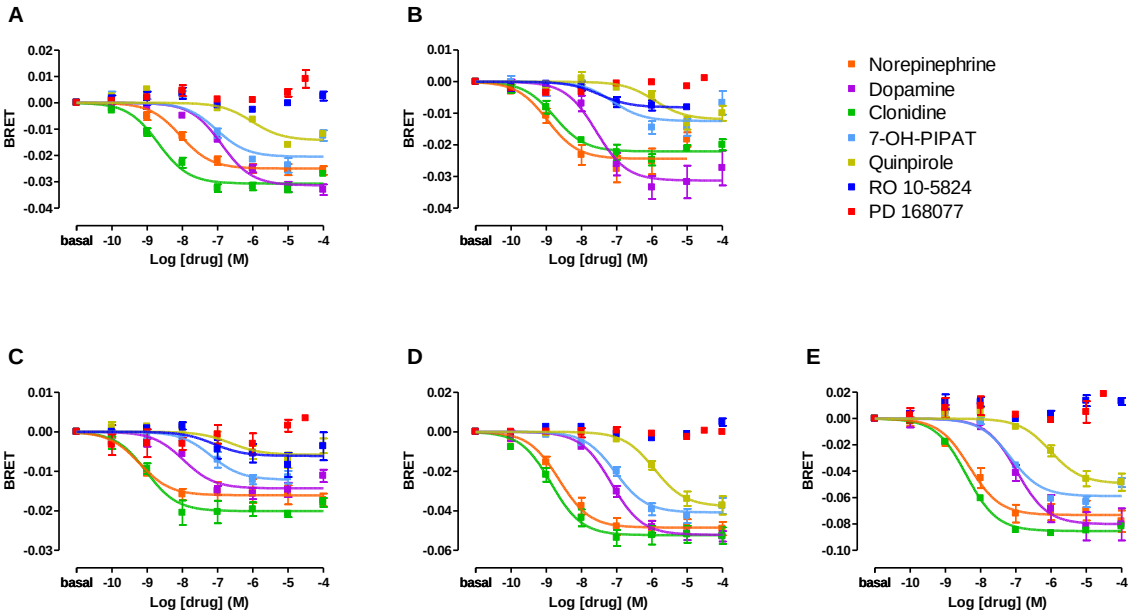

Figure 1. G protein activation-mediated by NE, DA, clonidine and D_2-like receptor ligands by α2AR. Concentration-response experiments of G protein activation by NE, DA, clonidine, 7-OH-PIPAT, Quinpirole, RO 10-5824 and PD-168077 mediated by α2AR in HEK-293T cells transiently transfected with α2AR, the G protein subunits Gαi1-RLuc (A), Gαi2-RLuc (B), Gαi3-RLuc (C), Gαo1-Rluc (D) or Gαo2-RLuc (E), γ2-mVenus and non-fused β1. Cells were treated with Coelenterazine H followed by increasing concentrations of one of the ligands. Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 3 to 11 experiments performed in triplicate (see Table 1 and 2 for EC_{50} and E_{max} values and statistical analysis).

Table 1. Potency of NE, DA, clonidine, 7-OH-PIPAT and quinpirole obtained from G-protein activation experiments mediated by α2AR and α2CR coupled to the different Gαi/o subtypes

Gα Subunit	Receptor	NE	DA	Clonidine	7-OH-PIPAT	Quinpirole	DA/NE
		EC_{50} (nM)					
Gαi1	α_{2A}	11 ± 2 **	170 ± 40	3 ± 1	80 ± 20 *	700 ± 250	15
	α_{2C}	90 ± 30	160 ± 50	6 ± 2	(8 ± 4)	ND	1.8
Gαi2	α_{2A}	1.3 ± 0.3 *	30 ± 3 **	2.0 ± 0.8	120 ± 6 *	(1000 ± 300)	23
	α_{2C}	0.4 ± 0.2	5 ± 3	3 ± 1	6 ± 1	(200 ± 100)	12.5
Gαi3	α_{2A}	0.6 ± 0.2	15 ± 5	1.0 ± 0.2	60 ± 20 *	(700 ± 400)	25
	α_{2C}	0.4 ± 0.2	30 ± 20	4 ± 1	5 ± 3	400 ± 100	2
Gαo1	α_{2A}	3.0 ± 0.5 *	80 ± 10 **	2.0 ± 0.4 *	100 ± 20 **	1300 ± 200 **	27
	α_{2C}	19 ± 6	126 ± 8	(12 ± 5)	20 ± 3	(230 ± 50)	7
Gαo2	α_{2A}	6 ± 1 *	100 ± 20	4.0 ± 0.2 **	66 ± 7 *	820 ± 80 ***	17
	α_{2C}	50 ± 10	140 ± 20	11 ± 2	19 ± 9	(100 ± 10)	2.6

Potency (EC_{50} values, in nM) of NE, DA, clonidine and D_2-like receptor ligands obtained from G-protein activation experiments mediated by α2AR and α2CR coupled to the different Gαi/o subtypes (Figs. 1 and 2). EC_{50} values were obtained from a sigmoidal concentration-response function adjusted by nonlinear regression analysis and are expressed as means ± S.E.M. of 2 to 11 experiments performed in triplicate. In parenthesis, values corresponding to experiments showing low efficacy, E_{max} lower than 50% (Table 2). DA/NE: ratio of EC_{50} values of DA and NE for each receptor and Gαi/o protein subtype. Statistical differences between α2AR and α2CR were calculated by non-paired, two- tailed Student's t test; *, ** and ***: $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

The prototypical non-selective α adrenoceptor agonist clonidine only showed a higher potency at α2AR than at α2CR adrenoceptors for Gαo1 and Gαo2 (Table 1). An additional difference as compared to NE was that clonidine behaved as a full agonist at α2AR and as a partial agonist at α2CR, except for Gαi2 and Gαi3 (Figs. 1 and 2 and Table 2). Intriguingly, the

level of efficacy of α2CR varied significantly with the associated Gαi/o protein subtypes, from no decrease for Gαi2 to a very significant loss of efficacy for Gαo1 (Table 2). Previous studies have already reported a partial agonism of clonidine at α_2 adrenoceptors, but with disparate results (Pohjanoksa et al., 1997; Kukkonen et al., 1998), which at least for α2CR could be explained by the Gαi/o protein subtypes involved.

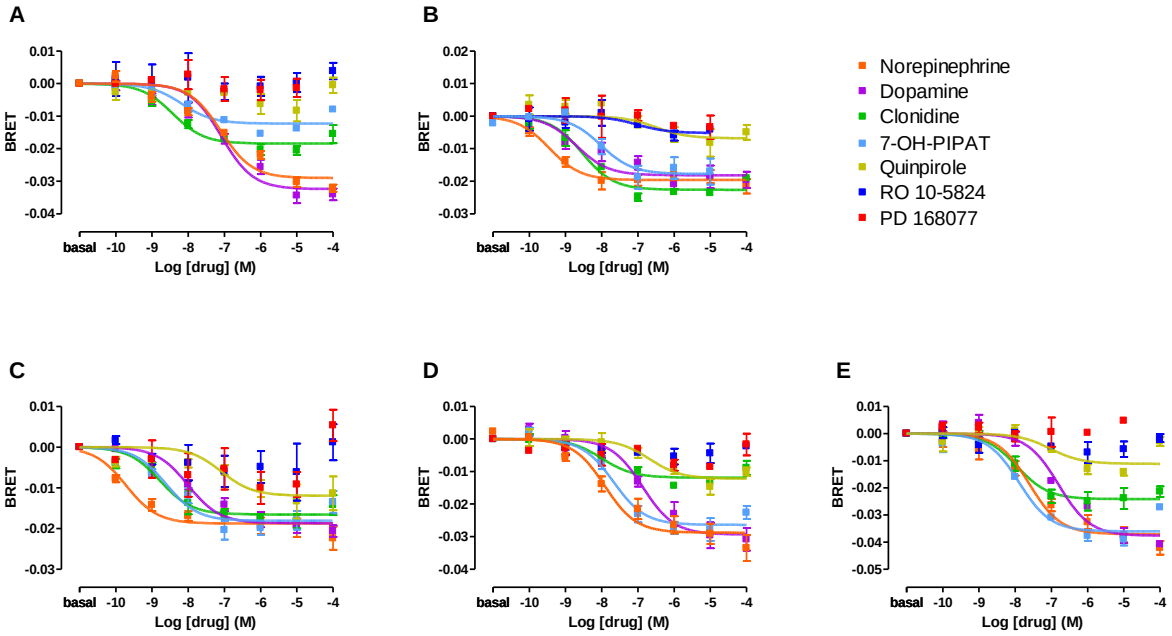

Figure 2. G protein activation-mediated by NE, DA, clonidine and D_2-like receptor ligands by α2CR. Concentration-response experiments of G protein activation by NE, DA, clonidine and D_2-like receptor ligands mediated by α2CR in HEK-293T cells transiently transfected with α2C receptor, the G protein subunits Gαi1-RLuc (A), Gαi2-RLuc (B), Gαi3-RLuc (C), Gαo1-RLuc (D) or Gαo2-RLuc (E), γ2-mVenus and non-fused β1. Cells were treated with Coelenterazine H followed by increasing concentrations of one of the ligands. Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 2 to 9 experiments performed in triplicate (see Table 1 and 2 for EC_{50} and E_{max} values and statistical analysis).

The non-selective D2R-D3R-D4R agonist quinpirole and the selective D3R agonist 7-OH-PIPAT also activated α2AR and α2CR, but with very different profiles (see Figs. 1 and 2 and Tables 1 and 2). 7-OH-PIPAT behaved as a potent full agonist at the α2CR adrenoceptor for most G αi/o subtypes. For both Gαo subtypes, the D3R agonist was as potent and efficacious α2CR agonist as NE. Only for Gαi1, 7-OH-PIPAT behaved as a very little efficacious α2CR agonist. 7-OH-PIPAT

also showed high efficacy at α2AR for most Gαi/o subtypes, except for Gαi2 (with half the efficacy than NE), with potencies similar to DA. Quinpirole showed a weak potency (submicromolar range) and behaved as a partial or full agonist depending on the Gαi/o subtype. At α2AR, quinpirole behaved as a partial agonist for Gi1, Gi2 and Gi3 and full agonist for Go1 and Go2, whereas at α2CR it behaved as a partial agonist for all G protein subtypes except for Gi3 (full agonist) and showed no activity when coupled with Gαi1. The specificity of the signal produced by quinpirole and 7-OH-PIPAT was addressed using the non-selective α_2 adrenoceptor antagonist yohimbine. As shown in Fig. 3, yohimbine completely blocked the full agonistic effect of 7-OH-PIPAT and the partial agonistic effect of quinpirole at both α2AR and α2CR (for Gαo1).

Table 2. Efficacy of NE, DA, clonidine, 7-OH-PIPAT and quinpirole obtained from G-protein activation experiments mediated by α_{2A} and α_{2C} adrenoceptors coupled to the different Gαi/o subtypes

Gα Subunit	Receptor	NE	DA	Clonidine	7-OH-PIPAT	Quinpirole
		E_{max} (% of NE values)				
Gαi1	α_{2A}	100 ± 5	120 ± 6 *	120 ± 5	94 ± 6	70 ± 2 *
	α_{2C}	100 ± 2	105 ± 5	64 ± 4 **	*41 ± 1* **	ND
Gαi2	α_{2A}	100 ± 10	120 ± 20	89 ± 6	55 ± 2 *	*47 ± 8* *
	α_{2C}	100 ± 7	80 ± 10	107 ± 4	90 ± 20	*46 ± 9* **
Gαi3	α_{2A}	100 ± 10	90 ± 10	112 ± 8	71 ± 6	*40 ± 9* **
	α_{2C}	100 ± 9	95 ± 6	85 ± 5	90 ± 10	80 ± 15
Gαo1	α_{2A}	100 ± 7	108 ± 6	110 ± 10	81 ± 9	78 ± 9
	α_{2C}	100 ± 10	100 ± 10	*41 ± 3* **	98 ± 7	*42 ± 3* **
Gαo2	α_{2A}	100 ± 9	110 ± 10	120 ± 2	85 ± 4	72 ± 8
	α_{2C}	100 ± 8	114 ± 6	70 ± 8 *	104 ± 4	*34 ± 6* **

Efficacy (E_{max} values, as percentage of NE values) of NE, DA, clonidine and D_2-like receptor ligands obtained from G-protein activation experiments mediated by α_{2A} and α_{2C} coupled to the different Gαi/o subtypes (Figure 1 and 2). E_{max} values were obtained from a sigmoidal concentration-response function adjusted by nonlinear regression analysis and are expressed as means ± S.E.M. of 2 to 11

experiments performed in triplicate. In italics, values of E_{max} lower than 50%. ND: not detectable. Statistical differences between NE and the other ligands for each receptor and Gαi/o protein subtype were calculated by one-way ANOVA, followed by Dunnett post hoc test; * and **: $p<0.05$ and $p<0.01$, respectively

Finally, the selective D4R agonists RO-105824 and PD-168077 did not produce a significant activation of α2AR or α2CR coupled to any of the Gαi/o subtypes, except for a small efficacy of RO-105824 at α2A for Gαi2 and Gαi3 (Figs. 1 and 2). To discard any possible binding of these ligands to α2AR and α2CR adrenoceptors, they were tested for their ability to modify the effect of clonidine. Surprisingly, RO- 105824, but not PD-168077, counteracted the respective full and partial agonistic effect of clonidine (1 µM) at the α2AR and α2CR coupled to Gαo1 (Fig. 3C-D). These results therefore disclosed a previously unknown additional role of the D4R agonist RO-105824, as a potent and low-efficient ligand for α_2 adrenoceptors.

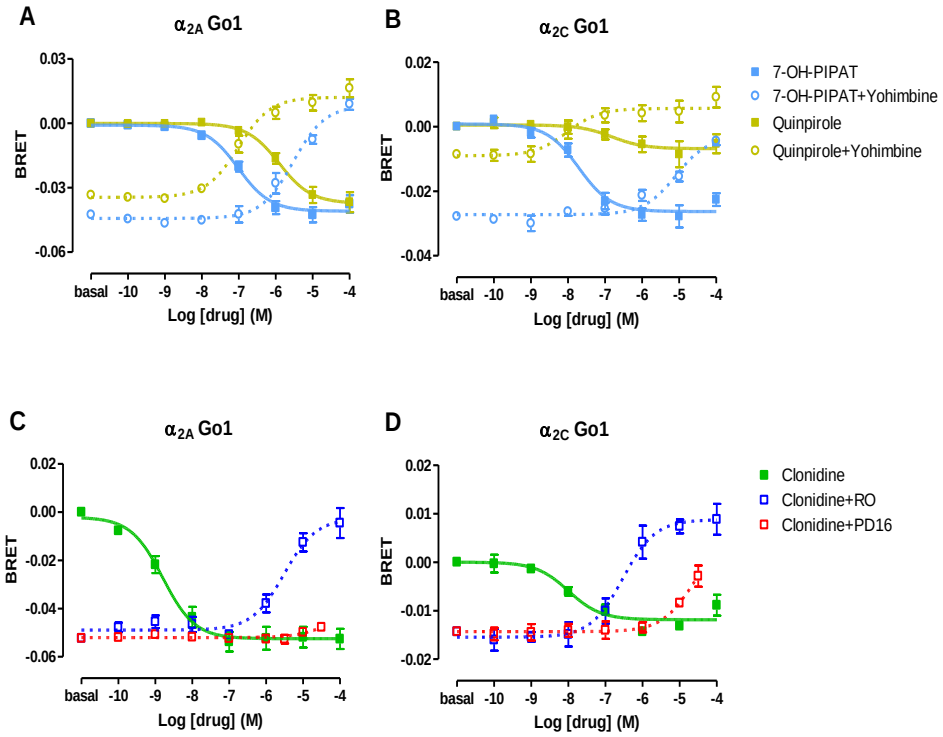

Figure 3. (A, B) Dose-dependent inhibition by increasing concentrations of the non-selective α_2 receptor antagonist yohimbine of Gαo1 protein activation induced by the D_2-like receptor agonists 7-OH-PIPAT (blue) or quinpirole (yellow) in HEK 293T cells transfected with α2AR (A) or α2CR (B), Gαo1-RLuc, γ2-mVenus and non-fused β1. Cells were treated with Coelenterazine H followed by the addition of 7-OH-PIPAT or quinpirole. Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of agonists were subtracted from the BRET values for each agonist concentration. (C, D) dose-dependent inhibition

by increasing concentrations of RO-105824 (dark blue) or PD-168077 (red) in cells transfected with α2AR (C) or α2CR (D), Gαo1-RLuc, γ2-mVenus and non-fused β1. BRET values in the absence of agonists were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 3 to 6 experiments performed in triplicate.

2.2 Binding of DA and DA receptor ligands to α_2 adrenoceptors in cortical and striatal tissue.

Searching for a correlate in brain tissue of the results obtained in transfected cells, we analyzed the ability of NE, DA, clonidine, quinpirole, 7-OH-PIPAT, RO-105824 and PD-168077 to displace the binding of the non-selective α_2 adrenoceptor antagonist [^{3}H]RX821002 from membrane preparations from sheep cortex and striatum (competitive inhibition experiments) (Fig. 4). See Material and Methods for description of the variables. Saturation experiments with [^{3}H]RX821002 for cortical and striatal tissue provided B_{max} values of 0.33 ± 0.02 and 0.13 ± 0.02 pmol/mg protein and affinity values (K_{DA1}) of 0.06 ± 0.01 and 0.07 ± 0.01 nM (n=4-8), respectively. This implies that the density of α_2 adrenoceptors in the cortex, which is mostly represented by α2AR, is three times higher than in the striatum, which expresses similar densities of both α2AR and α2CR adrenoceptors. K_{DB1}, K_{DB2} and D_{CB} values from competition experiments of [^{3}H]RX821002 with NE, DA, clonidine and the D_2-like receptor ligands are presented in Table 3.

Results were largely in agreement with the potency values obtained with G protein activation BRET assay, considering that cortical values should represent ligand binding parameters of α2AR, while striatal values represent combined ligand binding parameters for both α2AR and α2CR. Thus, in both tissues, NE, DA and clonidine showed high affinity for [^{3}H]RX821002 binding sites with an order of potency of clonidine>NE>DA (Fig. 4). The three ligands showed negative cooperativity (negative D_{CB} values). The affinity of NE was higher in the cortex than in the striatum, with higher striatal K_{DB1} and K_{DB2} values and higher D_{CB} values (stronger negative cooperativity) (Table 3). The affinity of DA was very similar in both tissues, with similar K_{DB1} values and a moderately but significantly higher K_{DB2} value in the striatum, resulting in similar D_{CB} values (Table 3). The affinity of clonidine was also higher in the cortex, with a significantly higher striatal K_{DB1} value and similar D_{CB} values (Table 3). The affinity of clonidine was also higher in the cortex, with a significantly higher striatal K_{DB1} value and similar

K_{DB2} and D_{CB} values (Table 3). In summary, the higher affinities of NE and clonidine in the cortex and similar affinities of DA in cortex and striatum also correlate with the differences in their respective potency values for α2AR and α2CR from the G protein activation BRET experiments.

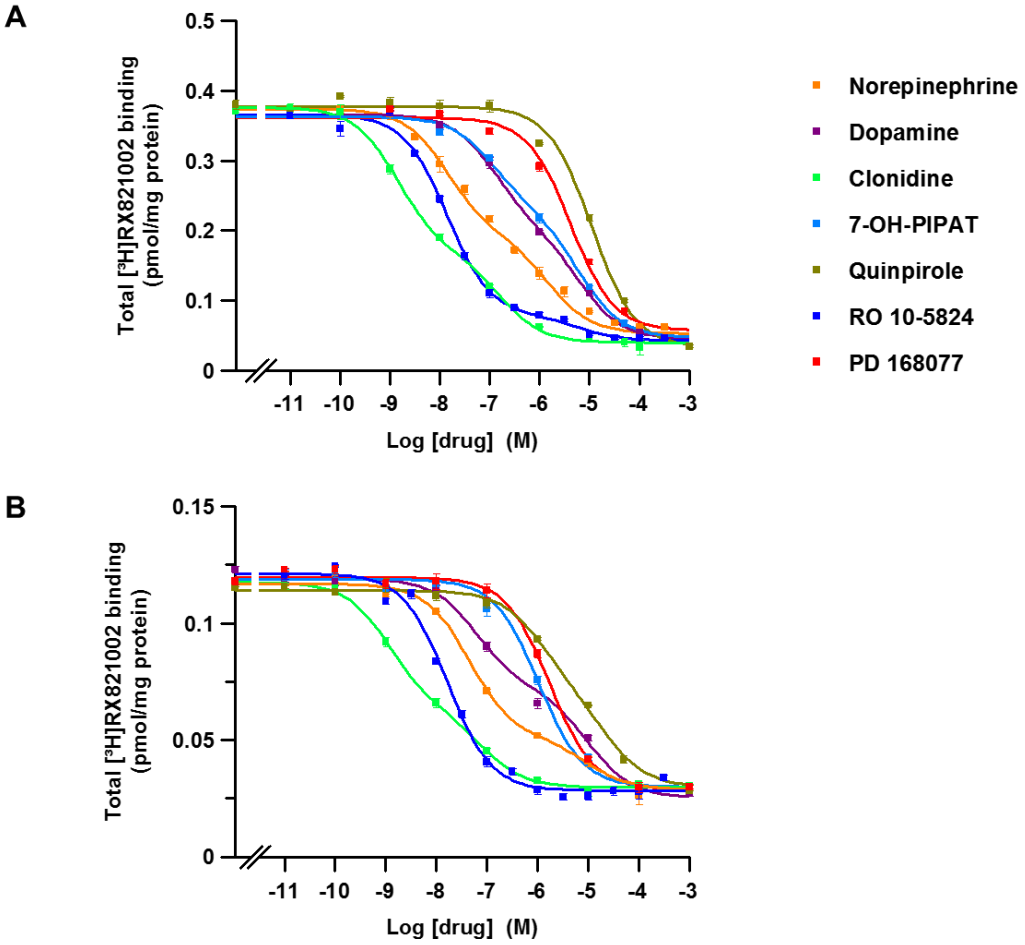

Fig 4. Competition binding experiment of [^{3}H]RX821002 *versus* NE, DA, Clonidine and D_2-like agonists. Representative competition curves of α_2 adrenoceptor antagonist [^{3}H]RX821002 total binding *versus* increasing concentrations of free competitors (NE, DA, clonidine, quinpirole, 7-OH-PIPAT, RO-105824 and PD-168077) in sheep brain cortical (A) and striatal (B) membranes. Experimental data were fitted to the two-state dimer receptor model equations, as described in the Material and Methods section. Values are mean ± S.E.M. from a representative experiment (n = 3) performed in triplicate.

7-OH-PIPAT and quinpirole also displaced [^{3}H]RX821002 binding with nanomolar and submicromolar affinities, respectively, which correlates with the potency values in the G protein activation BRET experiments (Table 3 and Fig. 4). Interestingly, 7-OH-PIPAT showed negative cooperativity in the cortex, but not in striatum. The only measurable affinity parameter of 7-OH-PIPAT in the striatum, K_{DB1}, was significantly higher than in the cortex and it was almost ten times lower than the cortical K_{DB2} value (Table 3). This would correlate with the predominantly lower potencies of 7-OH-PIPAT for α2CR *versus* α2AR in the G protein activation BRET experiments. Quinpirole also showed differences in the binding parameters between the cortex and striatum, such as a lower K_{DB1} value but negative cooperativity in the striatum. It would be however difficult to establish correlations with results from the G protein activation BRET experiments, where the often low efficacy of quinpirole might lead to inaccurate values (Table 1 in parenthesis and Table 2 in italics).

Finally, RO-105824 and PD-168077 also displaced [^{3}H]RX821002 binding from the cortex and striatum with high affinity (subnanomolar) and low affinity (submicromolar), respectively. No cooperativity (D_{CB} = 0) was obtained, which is usually the case for antagonists, except for RO-105824 in the cortex (D_{CB} = -4.3) (Table 3) (Casadó et al., 2007; Ferré et al., 2014). In fact, a small efficacy could be detected with RO-105824 for Gi2 and Gi3-mediated α2AR activation. Therefore, the binding experiments confirm the very high potency of the D4R agonist RO-105824 at α_2 adrenoceptors.

Table 3. **Competitive inhibition experiments of [^{3}H]RX821002 *versus* NE, DA, clonidine and D_2-like receptor ligands in sheep cortex and striatum.**

	CORTEX	**STRIATUM**
LIGAND	**Binding parameters**	
NE	K_{DB1}: 0.3 ± 0.2 * K_{DB2}: 250 ± 100 D_{CB}: -2.3	K_{DB1}: 0.8 ± 0,1 K_{DB2}: 5000 ± 3000 D_{CB}: -3.2
DA	K_{DB1}: 6.9 ± 0.2 K_{DB2}: 350 ± 10 * D_{CB}: -1.1	K_{DB1}: 6 ± 1 K_{DB2}: 1000 ± 200 D_{CB}: -1.6
Clonidine	K_{DB1}: 0.014 ± 0.003 * K_{DB2}: 40 ± 20 D_{CB}: -2.8	K_{DB1}: 0.036 ± 0.005 K_{DB2}: 20 ± 10 D_{CB}: -2.1
7-OH-PIPAT	K_{DB1}: 9 ± 2 ** K_{DB2}: 430 ± 80 D_{CB}: -1.1	K_{DB1}: 51 ± 6 D_{CB}: 0
Quinpirole	K_{DB1}: 530 ± 50 ** D_{CB}: 0	K_{DB1}: 110 ± 10 K_{DB2}: 2700 ± 400 D_{CB}: -0.8
RO-105824	K_{DB1}: 0.055 ± 0.003 *** $K_{DB2:}$ 4000 ± 2000 D_{CB}: -4.3	K_{DB1}: 0.42 ± 0.03 D_{CB}: 0
PD-168077	K_{DB1}: 100 ± 10 D_{CB}: 0	K_{DB1}: 83 ± 8 D_{CB}: 0

Binding parameters from competitive-inhibition experiments of [^{3}H]RX821002 *versus* NE, DA, clonidine and D_2- like receptor ligands in membrane preparations from sheep brain cortex and striatum (Fig. 4). K_{DB1}, K_{DB2} and D_{CB} values were obtained according to the two-state dimer model (see Results and ref. 26). K_{DB1} and K_{DB2} (in nM) are expressed as means ± S.E.M. of 3 experiments performed in triplicate. Statistical differences between affinity parameters of cortical *versus* striatal adrenoceptors were calculated by non-paired, two-tailed Student's t test; *, ** and ***: $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

2.3 α2AR and α2CR-mediated effects of NE and DA on adenylyl cyclase activity.

NE- and DA-induced Gi/o protein activation leads to the corresponding inhibition of adenylyl cyclase activation. We should however take into account that previous studies have shown that α2 adrenoceptors functionally couple not only to Gi/o proteins but also to Gs. (Fraser et al., 1989; Jones et al., 1991; Eason et al., 1992, 1994, 1995). Typically, the agonist concentrations necessary to elicit detectable stimulation of adenylyl cyclase are significantly higher than those for inhibition. Equivocal results were published by Zhang et al. (1999) (Zhang et al., 1999) when comparing the effect of NE and DA on forskolin-induced adenylyl cyclase activation. In their cell systems, NE seemed to predominantly activate Gs with α2AR and Gi with α2CR, while DA would predominantly activate Gi with both receptors, but at high micromolar concentrations. To find a functional correlate of the results obtained with NE and DA with the G protein activation BRET assay and with the radioligand binding experiments, we measured NE and DA-induced changes in adenylyl cyclase activity by measuring cAMP levels in intact cells transiently transfected with a2AR or a2CR, using CAMYEL, a kinase-dead Epac I–based cAMP BRET biosensor (Jiang et al., 2007). The biosensor detects the conformational changes in Epac that are induced upon its binding to cAMP. The conformational change triggered by an increase in cAMP induced by forskolin results in a decrease in BRET due to the relative orientation change between donor and acceptor. A decrease in forskolin-induced cAMP levels is therefore observed as an increase in BRET (Jiang et al., 2007). The same method was used to demonstrate the ability of NE to activate D_2-like receptors (Sánchez-Soto et al., 2016; Chapter 1 of the Results). As shown in Fig. 5, NE and DA produced inverted U-shaped concentration-response curves for both α2AR and α2CR-transfected cells. Therefore, NE and DA follow the same differential concentration-dependent effects on Gi/o and Gs- activation. As expected, the initial high potency effect, the decrease in adenylyl cyclase activity by NE and DA, provided apparent EC_{50} values that were qualitatively and quantitatively similar to those observed with the Gi/o activation BRET assay, as NE was more potent than DA at α2AR and α2CR (1.4 ± 0.2 and 6.2 ± 3 nM for NE and 91 ± 36 and 84 ± 20 nM for DA, respectively). Importantly, the NE- and DA-mediated Gi/o-Gs activation was blocked by the non-selective α_2 adrenoceptor antagonist yohimbine, confirming the receptor specificity of the signal. In addition, the Gi/o-dependent activation, but not the Gs-dependent activation, was blocked by the treatment with Pertussis Toxin. In

summary, adenylyl cyclase activity experiments confirm the results from radioligand binding and G protein activation experiments indicating that DA constitutes a potent α2AR or α2CR agonist.

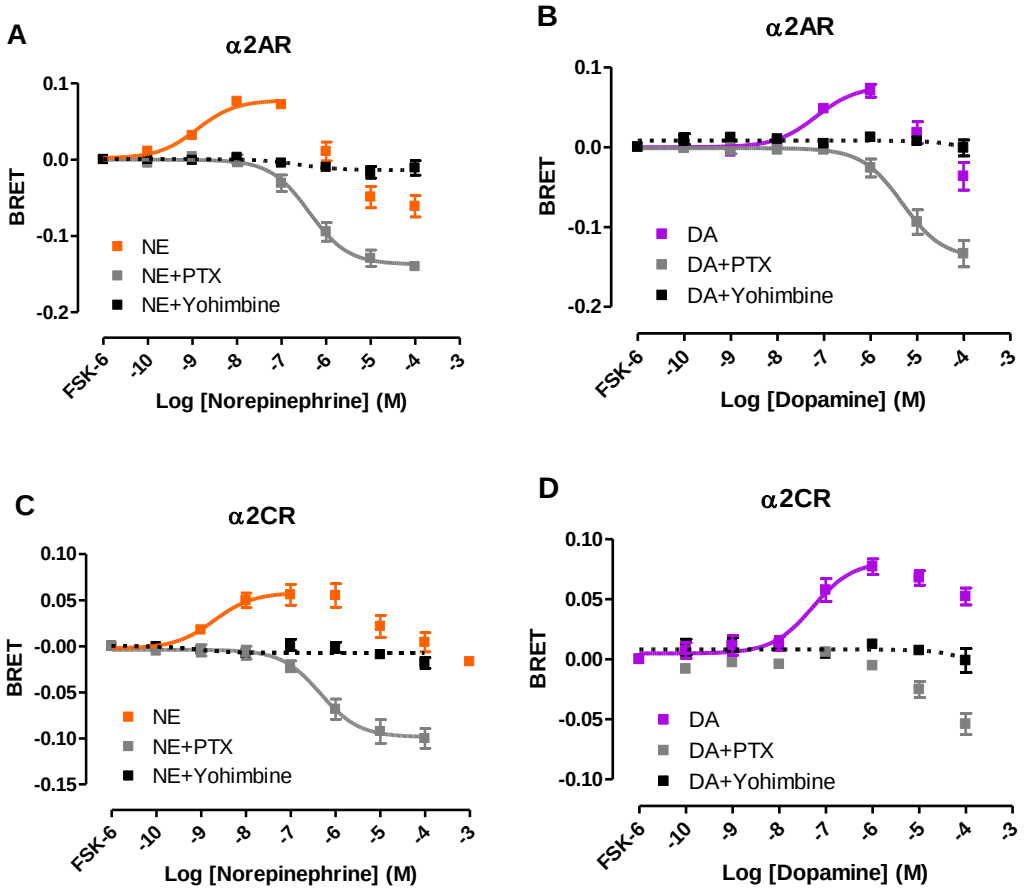

Figure 5. Concentration-response experiments of inhibition of forskolin-induced adenylyl cyclase activity by NE (orange) or DA (purple) mediated by α2AR (A, B) or α2CR (C, D) in HEK-293T cells transiently transfected with the CAMYEL sensor and one of the receptors. Cells were treated with forskolin (1 µM) for 10 minutes with or without the selective α_2 antagonist yohimbine (10 µM) followed by the addition of Coelenterazine H and increasing concentrations of NE or DA. After 10 minutes, BRET was measured as described in the Methods section. Values obtained with forskolin alone were subtracted from BRET values for each agonist concentration. Pertussis toxin (grey) was added 16 h before the experiment at a final concentration of 0.1 µg/ml. Data represent the mean ± S.E.M. of 3 to 7 experiments performed in triplicate.

2.4 Discussion

The present study demonstrates α_{2A} and α_{2C} adrenoceptors are significant targets for DA. The most conclusive demonstration comes from the results obtained with the G protein activation BRET assay, where the potencies of DA for α2AR and α2CR were found to be very similar or even higher than for some subtypes of D_2-like receptors (Sánchez-Soto et al., 2016; Chapter 1 of the Results). In particular, the EC_{50} values for α2AR and α2CR were consistently lower across all Gi/o protein subtypes as compared with the EC_{50} values for the predominant striatal D_2-like receptor D2L (Sánchez-Soto et al., 2016; Chapter 1 of the Results). Therefore, irrespective of the maximal concentration of extracellular NE that could be reached in the striatum, DA can reach sufficient extracellular concentration to activate α2AR and α2CR. In fact, striatal DA release sites are designed for transmitter spillover (Rice et al., 2011) and most striatal DA receptors are primarily extrasynaptic (Yung et al., 1995; Hersch et al., 1995), as obviously are the striatal adrenoceptors based on the mismatched low NE innervation (Ordway et al., 1993; Uhlen et al., 1997; Lindvall and Bjorklund, 1974; Swanson and Hartman, 1975; Aston-Jones, 2004). Although the specific functional role of the DA-sensitive α_2 adrenoceptors in neuronal striatal function remains to be established, a previous study suggests that they might mediate a modulatory role of the Gs/olf coupled striatal adenosine A_{2A} and dopamine D_1 receptors (Hara et al., 2010). The study of interactions might therefore provide new treatment approaches for Parkinson's disease and other basal ganglia disorders.

The possibility of DA-mediated activation of α2AR and α2CR adrenoceptors in extrastriatal areas should not however, be underestimated. Cortical α2AR are most probably able to be activated by DA, particularly in the prefrontal cortex, which receives a fairly dense DA innervation (Goldman-Rakic et al., 1992). In fact, there is recent evidence for the localization of α2AR in the cortical terminals from mesencephalic DA neurons (Castelli et al., 2016), which could play a role as "DA autoreceptors". But there is also evidence for the localization of both α2AR and α2CR adrenoceptors in the soma and dendrites of the mesencephalic DA cells of both substantia nigra and ventral tegmental area (Castelli et al., 2016; Lee et al., 1998). Apart from the NE input, these α2AR and α2CR adrenoceptors should be able to act as "DA autoreceptors" that control the non-synaptic somatodendritic DA release (Rice et al., 2011). Adding the present results to the previous study that also indicates a significant role of NE as

a Gi/o-coupled D_2-like receptor agonist (Sánchez-Soto et al., 2016; Chapter 1 of the Results), we could state that Gi/o-coupled adrenoceptors and DA receptors should probably be considered as members of one 'functional' family of catecholamine receptors. A general consideration from the DA and D_2-like receptor ligand sensitivity of cortical α2AR is that it should also be involved in the cognitive-enhancing effects associated with their activation, with possible implications for attention deficit hyperactivity disorder (Brennan and Arnsten, 2008).

The second major finding of the present study is that α2AR and α2CR adrenoceptors are also common targets for compounds previously characterized as D_2-like receptor ligands. Particularly striking was the ability of prototypical D3R and D4R agonists 7-OH-PIPAT and RO-105824 to bind with high affinity to α2AR and α2CR, which might call for revisiting results of previous studies using these compounds. RO-105824 behaved as a very potent α2AR and α2CR ligand with low intrinsic efficacy, while 7-OH-PIPAT disclosed a very different pharmacological agonistic profile between both receptors depending on the Gαi subtype (with loss of efficacy at α2AR for Gαi2 and at α2CR for Gαi1). In fact, both potency and efficacy dependence on the receptor and the Gαi/o protein subtype was the norm for all ligands, including the endogenous neurotransmitters. We already described that NE and DA show different receptor- and Gαi/o subtype-dependent potencies of D_2-like receptor-mediated G protein activation (Sánchez-Soto et al., 2016; Chapter 1 of the Results). The present results extend these findings to other receptors and to non-endogenous ligands, as well as to differences in intrinsic efficacy. Therefore, the present study demonstrates that the Gαi/o protein subtype is fundamental in conferring the specific pharmacological profile (potency and efficacy) of Gi/o-coupled catecholamine receptor ligands.

Our inferences from the pharmacological characterization of a compound in cell lines or native tissues are restrained by the lack of knowledge of the preferentially expressed Gαi/o subtype. Even though G proteins of the Gαs-Gαolf family do show contrasting brain expression pattern (Hervé, 2011), to our knowledge no clear region-specific pattern of mRNA expression for Gαi/o protein subtypes has been reported. Detailed characterization of the expression patterns for Gαi/o protein subtypes would then be central to determine their role in α2AR and α2CR activation and thus their possible specific targeting with Gαi/o subtype functionally selective compounds.

The results of this chapter are in the process of submission: **α_{2A}- and α_{2C}-Adrenoceptors as Targets for Dopamine and Dopamine Receptor Ligands.** Marta Sánchez-Soto*, Verònica Casadó-Anguera*, Hideaki Yano, Ning-Sheng Cai, Estefanía Moreno, Antoni Cortés, Vicent Casadó*, and Sergi Ferré*.

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Chapter 3.

Dopamine D_4 receptors resulting from the two most common gene polymorphic variants form different functional complexes with other catecholamine receptors

The dopamine (DA) D_4 receptor (D4R) belongs to the family of D_2-like receptors and its gene (*DRD4*) contains a large number of polymorphisms in its coding sequence (LaHoste et al., 1996). The most extensive polymorphism is found in exon 3, in a region that codes for the third intracellular loop (IC3) of the receptor (Van Tol, 1992). This polymorphism consists of a variable number of tandem repeats (VNTR), in which a 48-base pair sequence exists as a 2- to 11-fold repeat (Wang et al., 2004). The three most common variants contain 2, 4 and 7 TR and code for a D_4 receptor with 2, 4 and 7 repeats of a proline-rich sequence of 16 amino acids (D4.2R, D4.4R and D4.7R receptor). The *DRD4* gene with 4 TR constitutes the most frequent variant, with a global allelic frequency of 64%, followed by the variants with 7 TR (21%) and 2 TR (8%) (Chang et al., 1996). *DRD4* polymorphic variants have been suggested to be associated with numerous behavioral individual differences and neuropsychiatric disorders. The most reported association is the link between D4.7R and attention deficit hyperactivity disorder (ADHD) (Faraone et al., 2005; Li et al., 2006; Gizer et al., 2009) and also

substance use disorders (SUD) (McGeary, 2009; Belcher et al., 2014). The epidemiological data indicating significant clinical differences prompted to the search for functional differences associated with the products of these *DRD4* polymorphisms. Yet, although it was initially believed that D4.2R and D4.7R were less functional than D4.4R using some cell signaling assays, our recent study of the functional effect of DA at activating any of the three variants showed no significant differences in their ability to activate any of the five different Gi/o protein subtypes or to promote β arrestin-2 recruitment (Sánchez-Soto et al., 2016; Chapter 1 of the Results).

Very little was known about the role of the D4R in the brain and even less about the functional differences between the products of the different polymorphic variants until recent results from *in vivo* experiments obtained from our laboratory with knock-in mice containing the humanized D4.7R in its genome. This recent study demonstrates a key role of D4R in the control of corticostriatal transmission (Bonaventura et al., 2016). Interestingly, the D4.7 variant provided a gain of function of the receptor, compared to D4.4, increasing its ability to inhibit cortico-striatal glutamatergic neurotransmission. These results seemed at odds with those from previous studies, also performed in our laboratory, obtained *in vitro* with striatal slices from the same knock-in mice, where we reported they show a blunted MAPK signaling (González et al., 2012). In the same study, we also provided evidence for a specific decreased ability of the D4.7R to establish intermolecular and functional interactions with D_2 receptors (D2R) in transfected cells. Similar results were also obtained by another research group (Borroto-Escuela et al., 2011). It was concluded that D4.7R do not establish functional interactions with D2R (González et al., 2012).

It became therefore plausible that differential functional interactions with other receptors could provide an explanation for the differential attributes of D4.4R and D4.7R found at the level of brain function and dysfunction, in line with their clear lack of individual functional differences at the level of cell signaling (Sánchez-Soto et al., 2016; Chapter 1 of the Results). Consequently, we needed to investigate with more detail the signaling abilities of the D4.4R and D4.7R when forming heteromers with the D2R. We therefore used the recently introduced biophysical technique CODA-RET (Complemented Donor-Acceptor resonance energy transfer), a method that allows the study of the function of a signaling complex formed by two defined GPCRs and a subunit of the heterotrimeric G protein (Urizar et al., 2011). In

this assay, two complementary halves of Rluc are separately fused to each putative interacting receptors and YFP is fused to a specific Gα subunit. When the two receptors oligomerize the luciferase reconstitutes producing a functional donor for the energy transfer to YFP. We can then study the change in BRET values induced by ligands binding to either protomer (or both), which denotes G protein activation mediated by the oligomer. In addition to DA and NE we also analyzed the effect of dopaminergic ligands currently used for the treatment of Restless Legs Syndrome and Parkinson's disease: pramipexole, ropinirole and rotigotine which are well known D_2-like receptor agonists (Millan et al., 2002; Wood et al., 2015).

Although DA dysregulation in the prefrontal cortex and striatum is thought to be central to the neurobiology of ADHD, and the main pharmacological treatment involves psychostimulants, which increase DA levels in the brain, such as methylphenidate (Levy et al., 2001; Del Campo et al., 2011), converging evidence indicates that the pathophysiology of ADHD has multiple origins (Jensen, 2000; Donnelly, 2006; Soros, 2008; Pasini and D'Agati, 2009; Bock and Braun, 2011; Fusar-Poli et al., 2012; Sharma and Couture, 2014) and imbalance in the norepinephrine (NE) system is also involved (Zametkin and Rapoport, 1987; Arnsten et al., 1996). A similar situation to *DRD4* has emerged for the α_{2A} adrenoceptor (α2AR) gene (*ADRA2A*). Polymorphisms of this gene, particularly the 'G-allele' of a SNP in the promoter region (substituting C in position 1291; rs1800544), have been suggested to provide ADHD vulnerability as well as for symptoms of impulse control disorders (Roman et al., 2003; Park et al., 2005; Stevenson et al., 2005). However, a large meta-analysis did not find a consistent significant association (Gizer et al., 2009). Nevertheless, when considering an intermediate phenotype level (endophenotypes) (Belcher et al., 2014), a clear significant association could be established between *ADRA2A* polymorphisms (including the G-allele of the rs1800544) and the endophenotype impulsivity (Cummins et al., 2014). A recent study in rhesus monkeys has shown that guanfacine, a selective α2AR agonist currently approved for the symptomatic treatment of ADHD (Chan et al., 2016), significantly decreases impulsive choice (Kim et al., 2012). Finally, another recent study in humans showed a significant gene x drug interaction in which only cocaine users with risk *ADRA2A* polymorphisms demonstrated a significant increase in impulsive choice (Havranek et al., 2015).

In view of the common epidemiological and pharmacological involvement of D4R and α2AR in impulse control clinical disorders and, also, our results indicating the promiscuity of DA and NE in their ability to activate both Gi/o-coupled receptors, we explored the possibility of their ability to form functional interactions in transfected cells by CODA-RET. Again, D4.4R and D4.7 were compared and G protein activation BRET and CODA-RET techniques were used. In addition to DA and NE we also analyzed the effect of pramipexole, ropinirole and rotigotine.

3.1 D4.4R-D2R and D4.7R-D2R heteromer-mediated G protein activation

To study the effect of ligands on putative D4.4R-D2R and D4.7R-D2R heteromer-mediated signaling, HEK-293T cells were transfected with D4.4R or D4.7R fused to the N-terminal Rluc and D2R-fused to the C-terminal Rluc along with Gαi1-mVenus, β1 and γ2 and BRET was measured between receptor pair and Gα-subunit. The results were compared with those obtained with D2R, D4.4R and D4.7R homodimers, where one protomer of D2R, D4.4R or D4.7R is fused to the N-terminal Rluc and an equivalent protomer is fused to the C-terminal Rluc. Considering the effect of DA as the full response, NE mostly behaved as a full agonist and both DA and NE produced a dose-dependent D4.4R-D2R- and D4.7R-D2sR-mediated Gαi1-protein activation with potencies close to those observed for the D2R homodimer and with DA being between 10-60 more potent than NE (Fig 1A-1C; Table 1). As compared to the D2R-D2R homodimer, the potency of DA was slightly but significantly higher with the D4.7R-D2R heteromer and the relative efficacy of NE was significantly higher with the D4.4R-D2R heteromer (Table 1). A different pattern of activation was observed with D4.4R and D4.7R homodimers, where DA showed a significantly higher potency as compared to that obtained with the D2R homodimer, while the potency of NE was kept within the same range for D4.7R and was even lower for D4.4R, providing much higher EC_{50} NE/DA ratios (Fig 1D-1E; Table 1). The Gαi1-mVenus-associated signaling pattern of D2R and D4R homomers was then compared with that obtained from D2R and D4R expressing full Rluc, which does not assume any specific oligomerization state. The same as for homomers, DA had similar potencies for the two variants, D4.4R and D4.7R, which in both cases were significantly lower than for the D2R (Fig 2; Table 2). Surprisingly, NE had potencies for D4.4R and D4.7R that were relatively much closer to DA than for D4.4R and D4.7R homodimers, with much lower NE/DA ratios

(Tables 1 and 2). On the other hand, very similar NE/DA ratios were obtained with D2R and D2R-D2R homodimers.

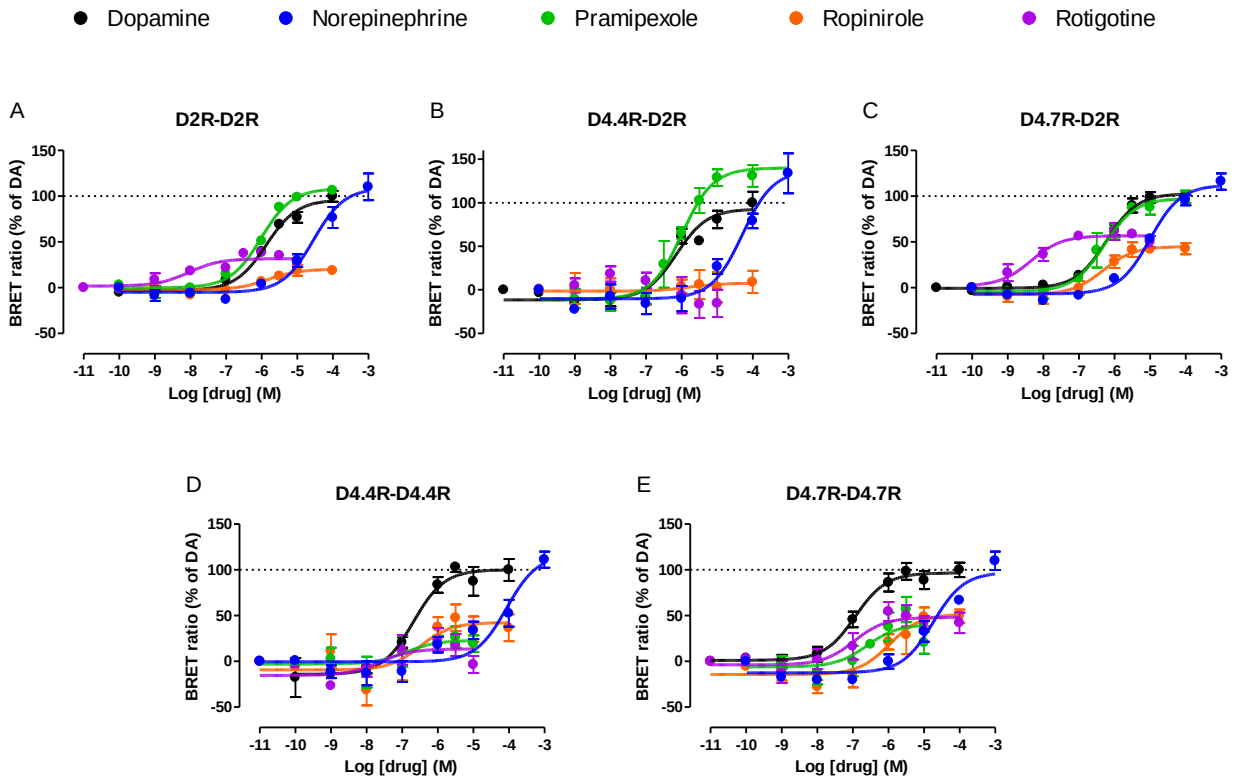


Figure 1. G protein activation mediated by DA, NE, pramipexole, ropinirole and rotigotine on D4R and D2R homo- and heteromers measured by CODA-RET. Concentration-response experiments of G protein activation by different ligands mediated by D2R-D2R, D4.4R-D2R, D4.7R-D2R, D4.4R-D4.4R and D4.7R-D4.7R in HEK-293T cells transiently transfected with D4.4R or D4.7R fused to L1 (N-terminal Rluc) and D2R-fused to L2 (C-terminal Rluc), Gαi1-mVenus, β1, and γ2. Cells were treated with Coelenterazine H followed by increasing concentrations of one of the ligands. Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 3 to 15 experiments performed in triplicate (see Table 1 for EC_{50} and E_{max} values and statistical analysis).

Table 1. Potency and efficacy of DA and NE obtained from CODA-RET experiments mediated by D2R and D4R homo- and heteromers.

		EC_{50}	E_{max}	NE/DA
D4.4R-D2R	**DA**	1000 ± 200	100 ± 6	60
	NE	60000 ± 10000	133 ± 9 **	
D4.7R-D2R	**DA**	700 ± 100 *	100 ± 4	14
	NE	10000 ± 900	109 ± 6	
D2R-D2R	**DA**	1600 ± 200	100 ± 4	19
	NE	30000 ± 5000	110 ± 10	
D4.4R-D4.4R	**DA**	240 ± 60 **	100 ± 10	282
	NE	70000 ± 50000	95 ± 10	
D4.7R-D4.7R	**DA**	130 ± 30 **	100 ± 5	325
	NE	40000 ± 15000	119 ± 6	

Potency (EC_{50} values, in nM) and efficacy (E_{max}) of DA and NE obtained from CODA-RET experiments mediated by D4R and D2R homo- and heteromers (Fig 1). EC_{50} values were obtained from a sigmoidal concentration-response function adjusted by nonlinear regression analysis and are expressed as means ± S.E.M. of 3 to 15 experiments performed in triplicate. E_{max} is expressed as percentage of DA. NE/DA: ratio of EC_{50} values of NE and DA for each receptor pair. Statistical differences between the EC_{50} of each receptor pair were calculated for DA and NE by one-way ANOVA followed by Dunnett post-hoc test against D2R-D2R; * and **: $p<0.05$ and $p<0.01$, respectively. Statistical differences in E_{max} between DA and NE were calculated by one-way ANOVA followed by Dunnett post-hoc test of each ligand against DA (see also Table 2); **: $p<0.01$.

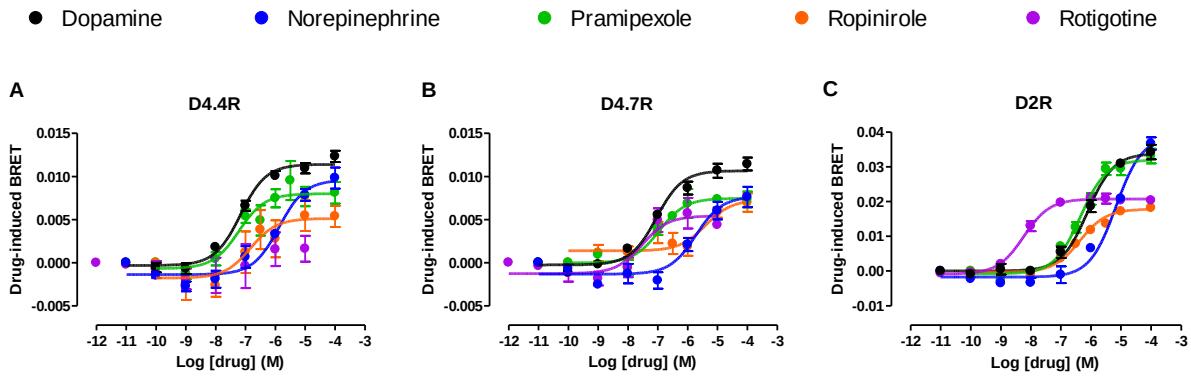


Figure 2. G protein activation mediated by DA, NE, pramipexole, ropinirole and rotigotine by D4.4R, D4.7R and D2R measured by G protein activation BRET. Concentration-response experiments of G protein activation by different ligands mediated by D4.4R, D4.7R and D2R in HEK-293T cells transiently transfected with D4.4R, D4.7R or D2R fused to full-length Rluc, Gαi1-mVenus, β1, and γ2. Cells were treated with Coelenterazine H followed by increasing concentrations of one

of the ligands. Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 3 to 10 experiments performed in triplicate (see Table 2 for EC_{50} and E_{max} values and statistical analysis).

Table 2. Potency and efficacy of DA, NE, pramipexole, ropinirole and rotigotine obtained from G protein activation BRET mediated by D4.4R, D4.7R and D2R.

	D4.4R			D4.7R			D2R		
	EC_{50}	E_{max}	NE/DA	EC_{50}	E_{max}	NE/DA	EC_{50}	E_{max}	NE/DA
DA	80 ± 15 **	100 ± 5	23	150 ± 50 **	100 ± 6	16	800 ± 100	100 ± 3	9
NE	1800 ± 400 **	96 ± 6		2400 ± 800 **	83 ± 9		7000 ± 1000	120 ± 4	
Pramipexole	60 ±20 **	76 ± 7 *		110 ± 20 **	68 ± 8 *		500 ± 100	97 ± 6	
Ropinirole	350 ± 150	62 ± 6 **		5000 ±2000	54 ± 8 **		480 ± 40	54 ± 2 **	
Rotigotine	NA	NA		35 ±15	60 ± 10 *		5 ± 2	70 ± 5 **	

Potency (EC_{50} values, in nM) and efficacy (E_{max}) of DA, NE, pramipexole, ropinirole and rotigotine obtained from G protein activation experiments mediated by D4.4R, D4.7R or D2R (Fig 2). EC_{50} values were obtained from a sigmoidal concentration-response function adjusted by nonlinear regression analysis and are expressed as means ± S.E.M. of 3 to 10 experiments performed in triplicate. E_{max} is expressed as percentage of DA. NE/DA: ratio of EC_{50} values of NE and DA for each receptor. Statistical differences between the EC_{50} of each receptor and each ligand were calculated by one-way ANOVA followed by Dunnett post-hoc test against D2R; **: $p<0.01$. Statistical differences in E_{max} between DA and the other ligands were calculated by one-way ANOVA followed by Dunnett post-hoc test of each ligand against DA; * and **: $p<0.05$ and $p<0.01$, respectively. NA, not adjusted.

Finally, the contribution of D2R and the D4R variants on the signaling by DA and NE on the D4.4R-D2R and D4.7R-D2R heteromers was analyzed by applying the selective D4R antagonist L745-870 and the D2R-D3R (not D4R) antagonist raclopride (Fig 3A-3B). For both neurotransmitters and for both D4.4R-D2R and D4.7R-D2R heteromers, raclopride (100 nM) produced an almost complete counteraction, while L745-870 (1 µM) slightly decreased the effect of DA and NE for D4.4R-D2R but not D4.7R-D2R heteromers (Fig 3C-3D).

In summary, we could confirm that there are no differences in the potency or efficacy of the endogenous neurotransmitters with D4.4R and D4.7R monomers or homodimers, but the association with D2R discloses differences between the two D4R variants. First,

catecholamine-induced Gi-mediated signaling by D4R-D2R heteromers is predominantly and completely dependent on the activation of D2R in D4.4R-D2R and D4.7R-D2R, respectively. D4R only plays a partial effect on the activation of D4.4R-D2R and no effect on the activation of the D4.7R-D2R heteromer. However, D4R variants play a differential modulatory role of DA- and NE-induced D4R-D2R heteromers-mediated signaling, with D4.4R increasing the efficacy of NE and D4.7R increasing the potency of DA probably through an allosteric modulation of D4.7R on D2R.

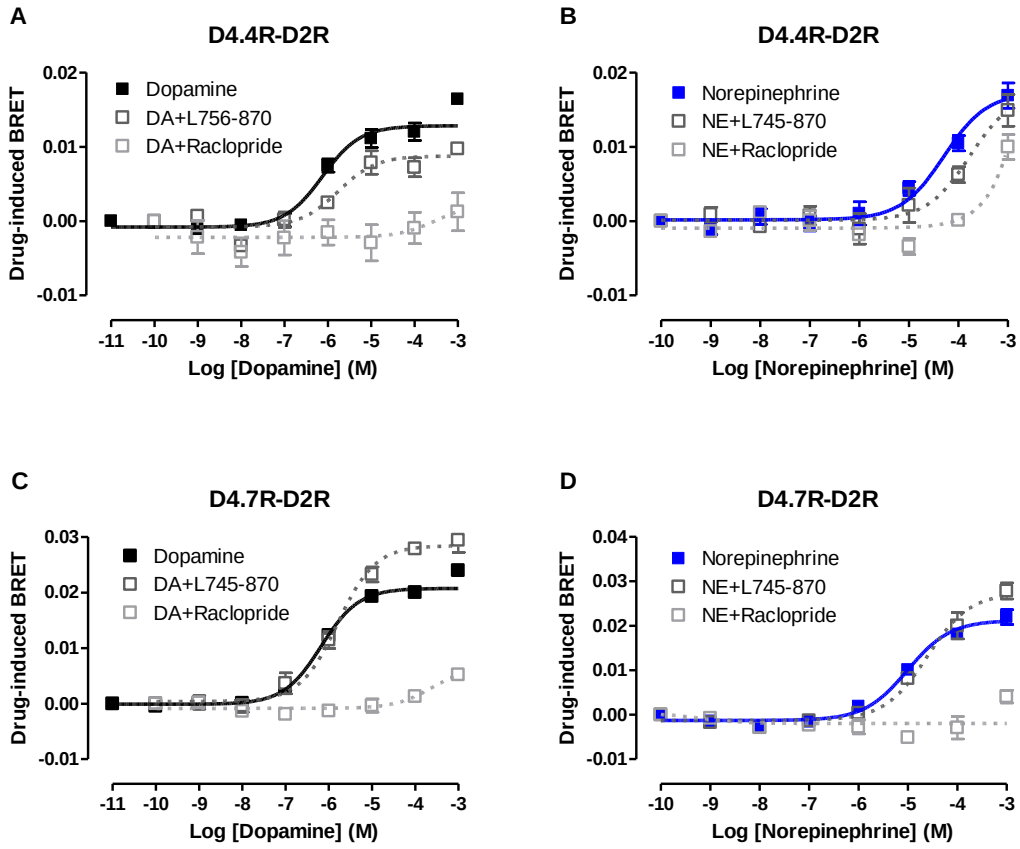


Figure 3. Inhibition of DA- and NE- mediated G protein activation by D4.4R-D2R (A, B) and D4.7R-D2R (C, D) heteromers measured by CODA-RET. Cells were pre-treated with 1 µM L745-870 or 100 nM raclopride for 10 minutes followed by the addition of Coelenterazine H and increasing concentrations of DA (black) or NE (blue). Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 3 to 15 experiments performed in triplicate.

An even more different D4R variant-dependent pattern emerged by analyzing the effects of the exogenous ligands on D4.4R-D2R and D4.7R-D2R heteromers. Qualitatively, pramipexole, ropinirole and rotigotine behaved with the D4.7R-D2R similarly than with the D2R homodimer: pramipexole showed full efficacy (in relation to DA) and ropinirole and rotigotine were partial agonists with an order of potency of rotigotine>pramipexole>ropinirole (Fig 1; Table 3). The same as for DA, the exogenous ligands were slightly more potent and efficacious for the D4.7R-D2R heteromer (only significant for ropinirole) than for the D2R homodimer (Fig 1; Table 3). On the other hand, for the D4.4R-D2R heteromers, pramipexole displayed higher efficacy than DA, while ropinirole and rotigotine were totally ineffective (Fig 1; Table 3). In addition, different to the endogenous neurotransmitters, a different profile could be observed with the exogenous ligands when considering D4R homodimers or the putatively monomeric conformation analyzed in the experiments without Rluc complementation. The most significant differences were that pramipexole was either a partial agonist (putative D4R monomer) or practically ineffective (D4R homodimer) and that rotigotine was ineffective in D4R monomers/homodimers (Fig 1 and 2; Tables 2 and 3). The same as for endogenous neurotransmitters, the same qualitative differences were observed with the profile of exogenous ligands for D2R homomers and with experiments with D2R expressing full Rluc (Fig 1 and 2; Tables 2 and 3).

In summary, we could establish for the first time differences in the potency or efficacy of the ligands between the D4.4R and D4.7R variants (monomers or homodimers). Furthermore, the association with D2R promoted additional differential modulations by D4R variants of ligand-induced D4R-D2R heteromers-mediated signaling. As compared with D2R homomers, D4.7R slightly increased, their potencies, while the D4.4R differentially modified their efficacy, increasing the efficacy of pramipexole, while blunting that of ropinirole and rotigotine.

Table 3. Potency and efficacy of pramipexole, ropinirole and rotigotine obtained from CODA-RET experiments mediated by D2R and D4R homo- and heteromers.

		EC_{50}	E_{max}
D4.4R-D2R	**Pramipexole**	1100 ± 200	125 ± 5 *
	Ropinirole	NA	
	Rotigotine	NA	
D4.7R-D2R	**Pramipexole**	440 ± 70	94 ± 5
	Ropinirole	600 ± 150 **	49 ± 5 **
	Rotigotine	3 ± 1 *	56 ± 7 **
D2R-D2R	**Pramipexole**	1200 ± 300	109 ± 2
	Ropinirole	2200 ± 500	28 ± 3 *
	Rotigotine	100 ± 20	31 ± 3 *
D4.4R-D4.4R	**Pramipexole**	NA	
	Ropinirole	400 ± 200 **	53 ± 4 *
	Rotigotine	NA	
D4.7R-D4.7R	**Pramipexole**	500 ± 350	65 ± 5 **
	Ropinirole	1300 ± 300	73 ± 8
	Rotigotine	190 ± 80	40 ± 10 **

Potency (EC_{50} values, in nM) and efficacy (E_{max}) of pramipexole, ropinirole and rotigotine obtained from CODA-RET experiments mediated by D4R and D2R homo- and heteromers (Fig 1). EC_{50} values were obtained from a sigmoidal concentration-response function adjusted by nonlinear regression analysis and are expressed as means ± S.E.M. of 3 to 15 experiments performed in triplicate. E_{max} is expressed as percentage of DA. Statistical differences between the EC_{50} of each receptor pair and for each ligand were calculated by one-way ANOVA followed by Dunnett post-hoc test against D2R-D2R; * and **: $p<0.05$ and $p<0.01$. Statistical differences in E_{max} between ligands were calculated by one-way ANOVA followed by Dunnett post-hoc test of each ligand against DA (see also Table 2); * and **: $p<0.05$ and $p<0.01$, respectively. NA, not adjusted.

3.2 D4.4R-α2AR and D4.7R-α2AR dimer-mediated G protein activation

CODA-RET was also used to demonstrate the ability of D4R to form functional interactions with α2AR and to evaluate possible differences in the effect of endogenous and exogenous ligands between D4.4R-α2AR- and D4.7R-α2AR-mediated signaling. HEK-293T cells were transfected with D4.4R or D4.7R fused to N-terminal Rluc and α2AR-fused to C-terminal Rluc along with Gαi1-mVenus, β1 and γ2 and BRET was measured between receptor pair and Gα-

subunit. The results could be compared with those obtained with α2AR homodimers, where one protomer of α2AR is fused to N-terminal Rluc and an equivalent protomer is fused to C-terminal Rluc. As demonstrated in our recent study on non-canonical activation of the α_2-adrenoceptors with DA (Sánchez-Soto et al., manuscript in preparation; Chapter 2 of the Results), DA was almost as potent as NE, although it showed a significantly lower efficacy (Fig 4; Table 4). The efficacy of DA was slightly increased with coupling to the D4.4R or D4.7R. Nevertheless, there was a selective increase in potency of DA over NE for the D4.4R-α2AR pair.

The Gαi1-mVenus-associated signaling pattern of α2AR homomers was then compared with that obtained from α2AR expressing full Rluc, which does not assume any specific oligomerization state in order to discard any possible artifact produced by the Rluc complementation. NE had high potency and efficacy for both α2AR and α2AR-α2AR (Fig 5A-B). DA had similar potency in both α2AR and α2AR-α2AR and the ratio NE/DA was almost identical in both cases. This supports the idea that α2AR forms homodimers and goes in line with the biphasic radioligand binding curves shown in Chapter 2 of the Results that considers receptors as dimers. On the other hand, the lower efficacy of DA on the α2AR-α2AR homomer contrasts with the almost full efficacy on α2AR (Fig 5B). An effect of the fusion protein on the normal function of the receptor cannot be discarded and more experiments are needed.

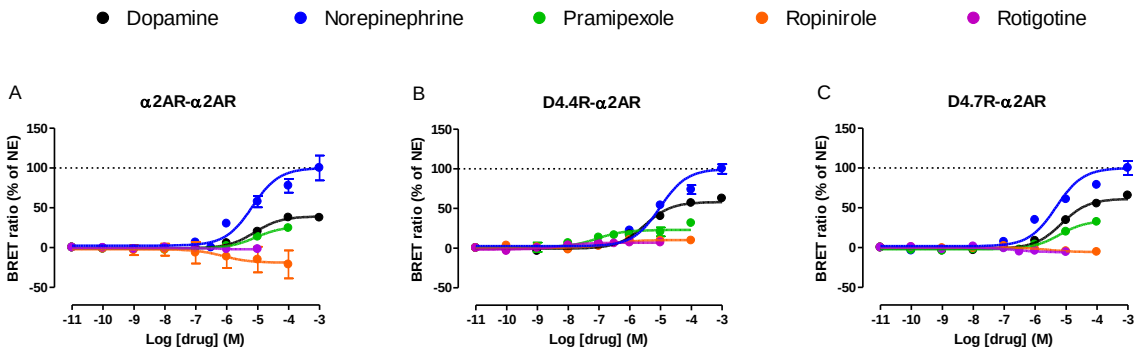
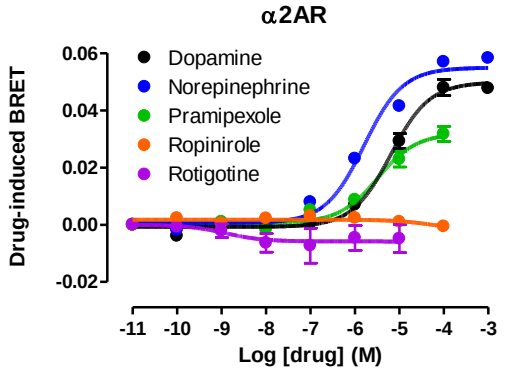


Figure 4. G protein activation mediated by DA, NE, pramipexole, ropinirole and rotigotine on D4R-α2AR and α2AR-α2AR measured by CODA-RET. Concentration-response experiments of G protein activation by different ligands mediated by α2AR-α2AR, D4.4R-α2A and D4.7R-α2AR in HEK-293T cells transiently transfected with D4.4R, D4.7R or α2AR fused to L1 (N-terminal Rluc) and α2AR-fused to L2 (C-terminal Rluc), Gαi1-mVenus, β1, and γ2. Cells were treated with Coelenterazine H followed by increasing concentrations of one of the ligands. Ligand-induced changes in BRET values were

measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 2 to 11 experiments performed in triplicate (see Table 4 for EC_{50} and E_{max} values and statistical analysis).

A



B

	α2AR		
	EC_{50}	E_{max}	NE/DA
DA	7500 ± 1000 **	95 ± 5	0.25
NE	1900 ± 500	100 ± 3	
Pramipexole	3270 ± 30	57 ± 5 **	
Ropinirole	NA	NA	
Rotigotine	NA	NA	

Figure 5. (A) Concentration-response and (B) potency and efficacy, of G protein activation mediated by DA, NE, pramipexole, ropinirole and rotigotine by α2AR measured by G protein activation BRET. (A) Concentration-response experiments of G protein activation by different ligands mediated by α2AR in HEK-293T cells transiently transfected with α2AR fused to full-length Rluc, Gαi1-mVenus, β1, and γ2. Cells were treated with Coelenterazine H followed by increasing concentrations of one of the ligands. Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 2 to 6 experiments performed in triplicate. (B) Potency (EC_{50} values, in nM) and efficacy (E_{max}) of DA, NE, pramipexole, ropinirole and rotigotine obtained from G protein activation experiments mediated by α2AR. E_{max} is expressed as percentage of NE. NE/DA: ratio of EC_{50} values of NE and DA. Statistical differences between the EC_{50} of ligands were calculated by one-way ANOVA followed by Dunnett post-hoc test against NE; **: $p<0.01$. Statistical differences between E_{max} of different ligands were calculated by one-way ANOVA followed by Dunnett post-hoc test of each ligand against NE; **: $p<0.01$. NA, not adjusted.

Table 4. Potency and efficacy of DA and NE obtained from CODA-RET experiments mediated by D4R-α2AR and α2AR-α2AR.

		EC_{50}	E_{max}	NE/DA
α2AR-α2AR	**DA**	8500 ± 700	54 ± 2 **	0.34
	NE	2900 ± 600	100 ± 9	
D4.4R-α2AR	**DA**	3500 ± 700 **	75 ± 5 **	1.2
	NE	4300 ± 700	100 ± 6	
D4.7R-α2AR	**DA**	6900 ± 700	77 ± 2 **	0.22
	NE	1500 ± 200	100 ± 4	

Potency (EC_{50} values, in nM) and efficacy (E_{max}) of DA and NE obtained from CODA-RET experiments mediated by α2AR-α2AR, D4.4R-α2AR or D4.7R-α2AR (Fig 4). EC_{50} values were obtained from a sigmoidal concentration-response function adjusted by nonlinear regression analysis and are expressed as means ± S.E.M. of 3 to 15 experiments performed in triplicate. E_{max} is expressed as percentage of NE. NE/DA: ratio of EC_{50} values of NE and DA for each receptor pair. Statistical differences between the EC_{50} of each receptor pair were calculated for DA and NE by one-way ANOVA followed by Dunnett post-hoc test against α2AR-α2AR; **: $p<0.01$. Statistical differences in E_{max} between DA and NE were calculated by one-way ANOVA followed by Dunnett post-hoc test of each ligand against NE *(see also Table 5); **: $p<0.01$.

The contribution of α2AR and the D4R variants on the signaling by DA and NE on the D4.4R-α2AR and D4.7R-α2AR pairs was analyzed by applying the selective D4R antagonist L745-870 and the α_2-adrenoceptor antagonist yohimbine (Fig 6). For both neurotransmitters and for both D4.4R-α2AR and D4.7R-α2AR pairs, yohimbine (1 μM) produced an almost complete counteraction, whereas L745-870 (1 μM) only significantly decreased the effect of DA and NE for D4.4R-α2AR but not D4.7R-α2AR pairs (Fig 6). In summary, the same as for D4.4R-D2R and D4.7R-D2R heteromers, we found that the association with α2AR discloses differences between the two D4R variants. First, catecholamine-induced Gi-mediated signaling by D4R-α2AR dimer is partially and completely dependent on the activation of α2AR in the D4.4R-α2AR and D4.7R-α2AR, respectively. Second, D4R only plays a partial effect on the G protein activation by D4.4R-α2AR and no effect on the activation by the D4.7R-α2AR pair. Then, D4R variants play a differential modulatory role of DA- and NE-induced D4R-α2AR-mediated

signaling, with D4.4R increasing the potency of DA *versus* NE. Finally, both D4.4R and D4.7R increase the efficacy of DA when they interact with α2AR.

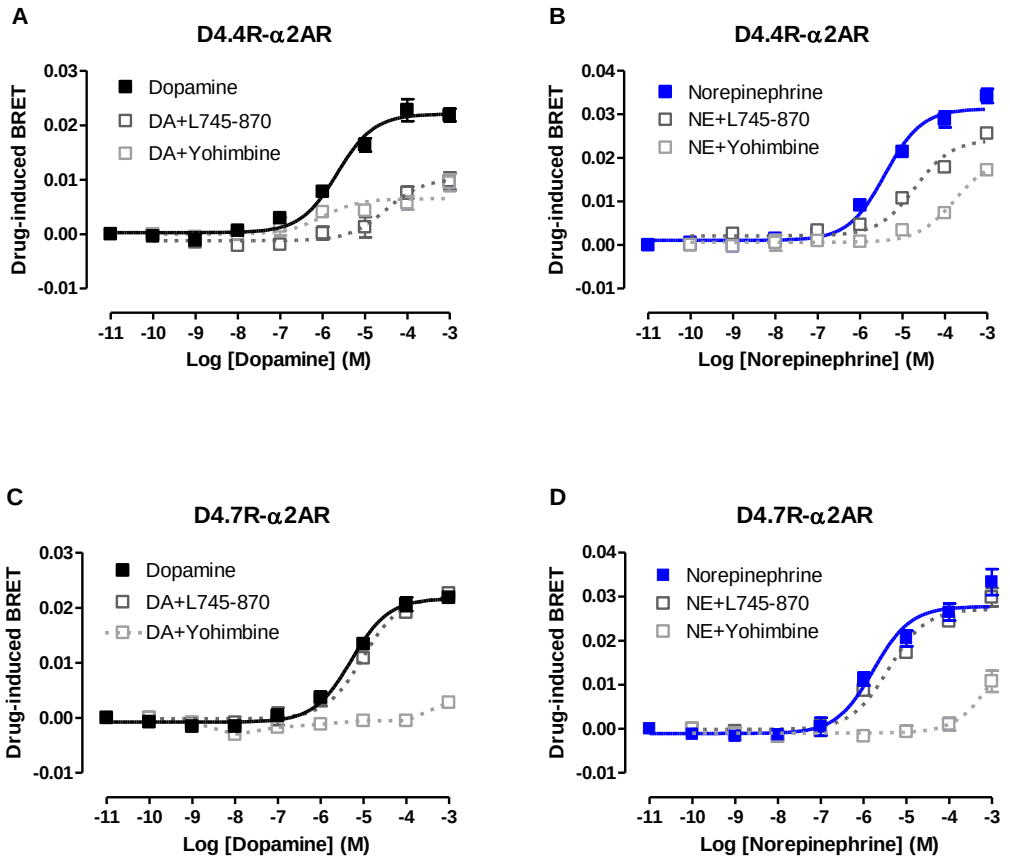


Figure 6. Inhibition of DA- and NE- mediated G protein activation by D4.4R-α2AR (A, B) and D4.7R-α2AR (C, D) dimers measured by CODA-RET. Cells were pre-treated with 1 µM L745-870 or 1 µM yohimbine for 10 minutes followed by the addition of Coelenterazine H and increasing concentrations of DA (black) or NE (blue). Ligand-induced changes in BRET values were measured as described in the Material and Methods section. BRET values in the absence of ligands were subtracted from the BRET values for each agonist concentration. Data were adjusted to a sigmoidal concentration-response function by nonlinear regression analysis and represent means ± S.E.M. of 4 to 11 experiments performed in triplicate.

Finally, the effects of pramipexole, ropinirole and rotigotine on the Gi-mediated signaling of D4.4R-α2AR and D4.7R-α2AR pairs were also analyzed and compared with their effects on α2AR homodimers (Fig 4; Table 5). The same pharmacological profile was observed for α2AR homodimers and D4.7R-α2AR pairs, a relatively low potency and efficacy of pramipexole and a lack of efficacy of ropinirole and rotigotine. Together with the same results obtained with DA and NE with α2AR homodimers and D4.7R-α2AR pairs and the total D4R-independent

D4.7R-α2AR pairs signaling, these results indicate that, although physically connected, D4.7R does not form functional signaling complexes with α2AR. On the other hand, pramipexole, ropinirole and rotigotine displayed high potency, although very partial efficacy, with D4.4R-α2AR pairs. Again demonstrating that interaction with other catecholamine receptors discloses important functional differences between the products of the two most common *DRD4* polymorphic variants. In addition, similar profile could be observed with the exogenous ligands when considering α2AR homodimers or the putatively predominant monomeric conformation analyzed in the experiments with full-length Rluc (Fig 5). Neither ropinirole nor rotigotine had any effect on G protein activation mediated by α2AR or α2AR-α2AR and pramipexole behaved as a partial agonist for monomers/homodimers (Fig 5).

Table 5. Potency and efficacy of pramipexole, ropinirole and rotigotine obtained from CODA-RET experiments mediated by D4R-α2AR and α2AR-α2AR pairs.

		EC_{50}	E_{max}
α2AR-α2AR	**Pramipexole**	9000 ± 3000	36 ± 2 **
	Ropinirole	NA	
	Rotigotine	NA	
D4.4R-α2AR	**Pramipexole**	140 ± 60 *	22 ± 2 **
	Ropinirole	350 ± 200	13 ± 2 **
	Rotigotine	7 ± 5	12 ± 2 **
D4.7R-α2AR	**Pramipexole**	5600 ± 650	44 ± 5 **
	Ropinirole	NA	
	Rotigotine	NA	

Potency (EC_{50} values, in nM) and efficacy (E_{max}) of pramipexole, ropinirole and rotigotine obtained from CODA-RET experiments mediated by α2AR-α2AR, D4.4R-α2AR or D4.7R-α2AR (Fig 4). EC_{50} values were obtained from a sigmoidal concentration-response function adjusted by nonlinear regression analysis and are expressed as means ± S.E.M. of 3 to 15 experiments performed in triplicate. E_{max} is expressed as percentage of NE. Statistical differences between the EC_{50} of each receptor pair and for each ligand were calculated by one-way ANOVA followed by Dunnett post-hoc test against α2AR-α2AR; * and ** $p<0.05$ and $p<0.01$, respectively. Statistical differences in E_{max} between ligands were calculated by one-way ANOVA followed by Dunnett post-hoc test of each ligand against NE; **: $p<0.01$. NA, not adjusted.

3.3 Discussion

The present results indicate that, first, there are no differences in the potency or efficacy of the endogenous neurotransmitters DA and NE with D4.4R and D4.7R monomers or homodimers, but the association with D2R or α2AR discloses differences between the two D4R variants.

Second, we have established for the first time differences in the potency or efficacy of exogenous ligands between the D4.4R and D4.7R variants. One example is the lack of effect of rotigotine on D4.4R compared to D4.7R where it behaves as a partial agonist. Furthermore, the association with D2R promoted additional differential modulations by D4R variants of ligand-induced D4R-D2R heteromers-mediated signaling. Interestingly, rotigotine behaved as a very potent agonist at D4.7R-D2R with a very low nanomolar affinity compared to D4.4R-D2R or D2R-D2R. This would suggest the need for genotyping in order to provide the most appropriate therapeutic agent for RLS and Parkinson's disease. In addition, the high affinity of rotigotine for this heteromer pair and the fact that is a drug approved in several countries would help in the treatment of other dopamine-based neuropsychiatric disorders by improving the efficacy and decreasing its side-effects.

Third, by using CODA-RET we have demonstrated the existence of functional Gi-mediated signaling complexes between α2AR and D4R *in vitro*, both being suggested to be involved in the pathophysiology and treatment of impulse control disorders (ADHD, substance use disorders). Finally, in transfected cells, α2AR can form functional complexes with D4.4R but not with the D4.7R variant, which is significantly associated with ADHD. These results go in line with previous experiments performed in our lab that suggested that D4.7R may not form functional heteromers with D2R (González et al., 2012).

Although to our knowledge, we are the first ones to suggest the putative interaction of α2AR and D4R, there is an obvious need to confirm this by a combination of different *in vitro, in situ* and *in vivo* techniques. As a matter of fact, this is currently one of the main focus in our lab and it will help in the understanding of the relationship between these receptors and neuropsychiatric disorders.

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V. GENERAL DISCUSSION

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GPCRs comprise the largest superfamily of proteins in the body. They are known as extremely versatile receptors for extracellular messengers as diverse as biogenic amines, purines and nucleic acid derivatives, lipids, peptides and proteins, odorants, pheromones, tastants, ions like calcium and protons, and even photons in the case of rhodopsin (Jacoby et al., 2006). GPCR signaling pathways regulate key biological functions such as cell proliferation, cell survival, angiogenesis and neurotransmission.

Catecholamines including DA and NE are widely distributed in the body and exert important physiological functions by serving as neurotransmitters or neuromodulators. Catecholamine neurotransmitter receptors belong to the GPCR superfamily and have been classically defined into subclasses of receptors with the name of the neurotransmitter with the highest affinity. However, there is compelling evidence indicating that DA and NE promiscuously interact with each other's receptors in some situations. One important example is the D4R which is known to be both a dopaminergic and noradrenergic receptor and act as an adrenergic receptor in certain parts of the brain (Lanau et al., 1997; Newman-Tancredi et al., 1997; Czermak et al., 2006; Cummings et al., 2010; Root et al., 2015). Yet, no clear evidence indicated that could also be the case for the other D_2-like receptors.

In general, NE and DA innervation coincides with the distribution of adrenergic and dopaminergic receptors, respectively, but this afferent-receptor coincidence is not always the case in the brain. Particularly in rodents, the cerebral cortex receives dense and widespread noradrenergic innervation, whereas dopaminergic terminals are more concentrated in the prefrontal cortex (Descarries et al., 1987; Séguéla et al., 1990). However, DA receptors are expressed throughout the cortex, and their localization exceeds that of the dopaminergic terminals (Lidow et al., 1989; Richfield et al., 1989; Goldsmith and Joyce, 1994; Wedzony et al., 2000).

In order to study the possible activation of D_2-like receptors by NE and how it compares to DA we used radioligand binding and three different BRET-based assays that allow measuring of adenylyl cyclase inhibition, activation of specific G protein subtypes and β-arrestin

recruitment. The results indicate that all D_2-like receptor subtypes (D2SR, D2LR, D3R, D4.2R, D4.4R and D4.7R) can bind with high affinity and be activated by NE. Importantly, their potencies, particularly for D3R and D4R, are closer than expected to the affinity for the adrenergic receptor α2AR. If D4R functions as an adrenergic receptor in some brain regions (Root et al., 2015), the other D_2-like receptors which can be expressed with higher density in some of those areas, should also be considered as functional adrenergic receptors. Furthermore, DA and NE concentrations in prefrontal cortex are comparable (Koob et al., 1975; Blank et al., 1979; Li et al., 1998), suggesting that actions of NE at cortical D_2-like receptors are likely functionally significant.

Since the first description of the human D4R variants (Van Tol, 1992), there has been a number of studies trying to decipher their differences in signaling as well as their association with neuropsychiatric disorders such as ADHD (). Initial studies seemed to indicate that D4.7R signals with less efficiency than the most common variant D4.4R (Asghari et al., 1995; Jovanovic et al.,1999) but our study clearly indicates that the three most common variants D4.2R, D4.4R and D4.7R do not have differences in their ability to activate Gi protein subtypes, inhibit adenylyl cyclase or recruit β-arrestin.

Our study also highlights the importance of the Gαi/o protein subunit involved in D4R activation. Thus, with the three main human D4R polymorphic variants DA was approximately 10 times more potent than NE at activating G proteins containing Gαi1, Gαi2, or Gαi3 subunits but it was only twice as potent at activating G proteins containing Gαo1 or Gαo2 subunits. Gαi/o protein subunit-dependent differences were also demonstrated in the potencies of DA and NE for D2SR, D2LR, and D3R.

The adrenergic α2AR is also a Gi/o-coupled receptor highly expressed in the prefrontal cortex (Wise et al., 1997) where D_2-like receptors are expressed, especially D2R and D4R (Missale et al., 1998). Our functional readouts of Gi activation, adenylyl cyclase inhibition, and β arrestin recruitment showed a relatively small separation between the potencies of NE for α2AR and D_2-like receptors (20-fold), verifying D_2-like receptors as potential signal transducers for NE.

Not only α2AR is expressed in the prefrontal cortex but it's also enriched in the striatum together with α2CR. The high density of striatal α2AR and α2CR prompted a fundamental

question in view of the well-known paucity of striatal noradrenergic terminals (Lindvall et al., 1975; Swanson and Hartman, 1975; Aston-Jones, 2004) and the concomitant low extracellular levels of striatal NE (Gobert et al., 2004). After showing that NE has a significant role as a DA D_2-like receptor agonist we decided to extend our study and characterize the effect of DA and other synthetic dopaminergic ligands on Gi/o-coupled adrenergic receptors. So in the second study, we revisited the possible dopaminergic function of α2AR and α2CR using the same methodology used previously, sensitive BRET-based techniques for the study of G protein activation or adenylyl cyclase activity in living cells. In addition, we also analyzed the ability of DA and NE to bind to α_2 adrenoceptors in cortical tissue, which predominantly expresses α2AR, and striatal tissue, which expresses both α2AR and α2CR adrenoceptors.

The most conclusive data comes from the G protein activation BRET assay, where the potencies of DA for α2AR and α2CR were found to be very similar or even higher than for some subtypes of D_2-like receptors. Therefore, irrespective of the maximal concentration of extracellular NE that could be reached in the striatum, DA can reach sufficient extracellular concentration to activate α2AR and α2CR. By combining the first and the second study we could state that Gi/o-coupled adrenoceptors and dopaminergic receptors should probably be considered as members of one 'functional' family of catecholamine receptors.

The second major finding of the present study is that α2AR and α2CR are also common targets for compounds previously characterized as D_2-like receptor ligands. Particularly striking was the ability of prototypical D3R and D4R agonists 7-OH-PIPAT and RO-105824 to bind with high affinity to α2AR and α2CR, which might call for revisiting results of previous studies using these compounds. Similar to the previous study, both potency and efficacy dependence on the receptor and the Gαi/o protein subtype was the norm for all ligands, including the endogenous neurotransmitters. Therefore, the first and the second study demonstrate that the Gαi/o protein subtype is fundamental in conferring the specific pharmacological profile (potency and efficacy) of Gi/o-coupled catecholamine receptor ligands. Detailed characterization of the expression patterns for Gαi/o protein subtypes would then be central to determine their role in α2AR and α2CR activation and thus their possible specific targeting with Gαi/o subtype functionally selective compounds.

To find a functional correlate of the results obtained with NE and DA with the G protein activation BRET assay and with the radioligand binding experiments, we measured NE and DA-induced changes in adenylyl cyclase activity by using CAMYEL, a BRET-based biosensor. Both ligands produced inverted U-shaped concentration-response curves for both α2AR and α2CR-transfected cells that correlate with a dual Gi/o-Gs effect. This goes in line with previous studies showing that α2 adrenoceptors functionally couple not only to Gi/o proteins but also to Gs (Fraser et al., 1989; Jones et al., 1991; Eason et al., 1992, 1994, 1995).

The role of D4R in the brain is not completely known, let alone the functional differences between the D4R variants. However, recent results in our laboratory demonstrated a key role of D4R in the control of corticostriatal transmission and a gain of function of D4.7R compared to D4.4R (Bonaventura et al., 2016). These results seem at odds with our results described above in which there is a lack of individual functional differences at the level of cell signaling. It became therefore plausible that differential functional interactions with other receptors could provide an explanation for the differential attributes of D4.4R and D4.7R found at the level of brain function and dysfunction described previously (Borroto-Escuela et al., 2011; Gonzalez et al., 2012b). Consequently, we needed to investigate with more detail the signaling abilities of the D4.4R and D4.7R when forming heteromers with D2R. It is now well accepted that GPCRs form homo- and heterodimers or high order oligomers at the membrane level. These protein-protein interactions implicate important changes in the pharmacological and functional properties of these receptors, and characterizing them is fundamental to understand their role in the brain as well as for their therapeutic implications.

A similar situation to the D4R gene has emerged for the α2AR gene. Polymorphisms of this gene have been suggested to provide ADHD vulnerability as well as for symptoms of impulse control disorders (Roman et al., 2003; Park et al., 2005; Stevenson et al., 2005). In view of the common epidemiological and pharmacological involvement of D4R and α2AR in impulse control disorders (ADHD, substance use disorder) and, also, our results indicating the promiscuity of DA and NE in their ability to activate both Gi/o-coupled receptors, we explored the possibility of their ability to form functional interactions in transfected cells by CODA-RET.

Therefore, in the third study we compared D4.4R and D4.7R by G protein activation BRET and CODA-RET techniques. CODA-RET is a BRET-based assay that allows the study of G protein

activation mediated by a known pair of receptors forming a complex. In addition to DA and NE we also analyzed the effect of three compounds approved for the treatment of RLS and Parkinson's disease, the D_2-like agonists pramipexole, ropinirole and rotigotine.

The results indicate that, first, there are no differences in the potency or efficacy of the endogenous neurotransmitters DA and NE with D4.4R and D4.7R monomers or homodimers (as expected from the results of the first part), but the association with D2R or α2AR discloses differences between the two D4R variants. Second, we have established for the first time differences in the potency or efficacy of exogenous ligands between the D4.4R and D4.7R variants. One example is the lack of effect of rotigotine on D4.4R compared to D4.7R where it behaves as a partial agonist or the very low nanomolar affinity at D4.7R-D2R compared to D4.4R-D2R or D2R-D2R. This would suggest the need for genotyping in order to provide the most appropriate therapeutic agent for RLS and Parkinson's disease. Third, by using CODA-RET we have demonstrated the existence of functional Gi-mediated signaling complexes between α2AR and D4R *in vitro*, both being suggested to be involved in the pathophysiology and treatment of impulse control disorders. Importantly, in transfected cells, α2AR can form functional complexes with D4.4R but not with the D4.7R variant, which is significantly associated with ADHD. These results go in line with previous experiments performed in our lab that suggested that D4.7R may not form functional heteromers with D2R (González et al., 2012b).

VI. CONCLUSIONS

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The conclusions derived from **chapter 1**, corresponding to aim 1 of this thesis are:

- Norepinephrine binds and activates dopamine D_2-like receptors with high affinity as it has been shown for radioligand binding, adenylyl cyclase inhibition, G-protein activation, and β-arrestin recruitment.

- D4R receptor variants behave functionally similar as shown with the different effector-specific BRET assays. We can hypothesize that their differences might arise from their heteromerization with other receptors.

- Application of BRET techniques that allow measuring activation of specific G protein subtype has provided evidence for a significant differential dependence on Gαi/o protein subunits on the ability of both dopamine and norepinephrine to activate dopamine D_2-like receptors.

- In summary, dopamine D_2-like receptors should be considered targets for norepinephrine, especially since their potencies, particularly for dopamine D3R and D4R, are only ∼20-fold lower than for α_{2A}-adrenoceptor activation.

The conclusions derived from **chapter 2**, corresponding to aim 2 of this thesis are:

- α2AR and α2CR are significant targets for the neurotransmitter dopamine. Surprisingly, the potencies for α2AR and α2CR adrenoceptors were found to be very similar or even higher than for some subtypes of D_2-like receptors. This could have important implications for the understanding of the role of α_2-adrenoceptors in the striatum and their possible activation by dopamine.

- α_{2A}- and α_{2C}-adrenoceptors are also common targets for synthetic compounds previously characterized as D_2-like receptor ligands. Particularly striking was the ability of the D3R and D4R receptor agonist 7-OH-PIPAT and RO-105824 to bind with high affinity to α2AR and α2CR.

- Both potency and efficacy dependence on the receptor and the Gαi/o protein subtype was the norm for all ligands, including the endogenous neurotransmitters.

- Norepinephrine and dopamine produced inverted U-shaped concentration-response curves for both α_{2A}- and α_{2C}-adrenoceptors-transfected cells. This NE- and DA-mediated Gi/o-Gs activation depends on the concentration of the ligand: Gi/o-mediated effects at lower concentration and Gs-mediated effects at higher concentration.

Therefore, two main conclusions can be drawn from the first and second chapter:

1. Gi/o-coupled adrenoceptors and dopamine receptors should probably be considered as members of one “functional” family of catecholamine receptors.

2. The Gαi/o protein subtype is fundamental in conferring the specific pharmacological profile (potency and efficacy) of Gi/o-coupled catecholamine receptor ligands.

The conclusions derived from **chapter 3**, corresponding to aim 3 of this thesis are:

- There are no differences in the potency or efficacy of the endogenous neurotransmitters dopamine and norepinephrine with D4.4R and D4.7R monomers or homodimers, but the association with D2R or α2AR discloses differences between the two D4R variants.

- We have established for the first time differences in the potency or efficacy of exogenous ligands between the D4.4R and D4.7R variants. Furthermore, the association with D2R promoted additional differential modulations by D4R variants of ligand-induced D4R-D2R heteromers-mediated signaling.

- Rotigotine is a very potent agonist for D4.7R-D2R compared to D4.4R-D2R or D2R-D2R. This could have implications for the treatment of RLS and Parkinson's disease.

- We have demonstrated the existence of intermolecular and functional interactions between α2AR and D4R, both being suggested to be involved in the pathophysiology and treatment of impulse control disorders (ADHD, substance use disorders).

- α2AR forms functional complexes with D4.4R but not with the D4.7R variant, which is significantly associated with ADHD.

VII. BIBLIOGRAPHY

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VIII. RESUM EN CATALÀ

VIII. RESUM EN CATALÀ

Activació dels receptors de catecolamines per neurotransmissors no canònics

Capítol 1: Evidència de l'activació per neurotransmissors no canònica: la noradrenalina com agonista del receptor D_2 de dopamina.

INTRODUCCIÓ

La dopamina és un important neurotransmissor implicat en la regulació d'aspectes importants de la funció cerebral que inclouen el moviment, la motivació, la memòria i l'addicció. S'uneix i activa dos famílies de receptors acoblats a proteïnes G (G protein-coupled receptors, GPCRs): els receptors D_1-like i els D_2-like. Els D_1-like estan formats pels receptors D_1 i D_5 mentre que els receptors D_2-like estan formats per D_2, D_3 i D_4. En general, la innervació dopaminèrgica coincideix amb la distribució dels receptors de dopamina, però no és sempre cert. El còrtex cerebral rep una gran innervació noradrenèrgica i malgrat que les terminacions dopaminèrgiques estan més concentrades al còrtex (Descarries et al., 1987; Séguéla et al., 1990), la localització dels receptors de dopamina excedeix la de les terminacions dopaminèrgiques. Una possible explicació ja descrita pel receptor D4 de dopamina (D4R) és la possibilitat de que els receptors dopaminèrgics puguin ser activats per noradrenalina (Lanau et al., 1997; Newman-Tancredi et al., 1997; Rivera et al., 2008; Cummings et al., 2010; Root et al., 2015). Tot i així, no hi ha evidències de que això sigui cert pels altres receptors D_2-

like. El D4R té un gran nombre de polimorfismes en la seva seqüència, un d'ells al tercer exó que codifica pel tercer loop intracellular del receptor. Es tracta d'un *variable number of tandem repeats* (VNTR), una seqüència de 48 bp que es repeteix de 2 a 11 vegades. Les tres variants al·lèliques més freqüents a la població humana són les variants amb 2, 4 i 7 repeticions en tàndem (D4.2R, D4.4R i D4.7R). Una d'aquestes variants, la D4.7, s'ha associat repetidament amb desinhibició, la qual és un endofenotip de TDAH (transtorn per dèficit d'atenció i hiperactivitat) i abús de drogues. Tot i així, no s'han trobat diferències clares en els productes de les variants polimòrfiques del gen del D4R. Per aquestes raons es va formular el següent objectiu:

> **Objectiu 1**: estudiar la possible activació dels receptors D_2-like (incloent les tres variants més prevalents del D4R) per noradrenalina mitjançant estudis d'unió de radiolligands i assajos funcionals basats en BRET.

RESULTATS

Mitjançant assajos d'unió de radiolligands en cèl.lules HEK-293 transfectades establement es van comparar les afinitats de la dopamina (DA) i la noradrenalina (NE) pels següent receptors: D2SR (D2-short), D2LR (D2-long), D3R i D4.4R. Els resultats obtinguts d'assajos de competició entre [^{3}H]7-OH-DPAT i DA o NE demostren que no només el D4R sinó que també els altres receptors dopaminèrgics tenen bona afinitat per la NE, amb una separació respecte la DA de només 4 (D2L) a 15 (D3R) vegades.

Els receptors D_2-like s'acoblen a Gi i inhibeixen l'adenilat ciclasa (AC) produint una baixada en l'AMPc. Per tal de comprovar els resultats d'unió de radiolligands, vam decidir estudiar la inhibició de l'AC mitjançada pels receptors D_2-like quan són activats per NE. Per fer això es va utilitzar el CAMYEL, un biosensor basat en BRET que permet mesurar els nivells d'AMPc intracel·lulars. Es van transfectar cèl·lules amb un dels receptors (D2SR, D2LR, D3R, D4.2R, D4.4R o D4.7R) juntament amb CAMYEL i es va comparar l'efecte de la DA i la NE en la inhibició de l'AC i conseqüent baixada de l'AMPc. Molt similar als experiments d'unió de radiolligands, tant la DA com la NE produeixen baixades d'AMPc i proporcionen valors de EC_{50} més baixos per D3R comparats amb D2LR o D2SR i lleugerament menors per D2SR que per D2LR. No es

van trobar diferències en els valors de EC_{50} per DA o NE en cèl·lules transfectades amb D4.2R, D4.4R o D4.7R.

Les proteïnes Gi es divideixen en 5 subtipus: Gi1, Gi2, Gi3 i les splice variants Go1 i Go2. Utilitzant BRET d'activació de proteïna G vam comparar la capacitat de DA i NE per activar els diferents subtipus de proteïnes Gi mitjançant l'activació de receptors D_2-like. Aquest assaig de BRET consisteix en l'ús de subunitats alfa de la G proteïna fusionades amb Rluc i subunitat gamma fusionada amb Venus (variant de la YFP). Quan el receptor és activat, produeix un canvi conformacional en la proteïna G que dóna lloc a una baixada en els valors de BRET. Utilitzant aquest sistema es va analitzar l'efecte de la DA i la NE en l'activació dels receptors D2SR, D2LR, D3R, D4.2R, D4.4R i D4.7R. En general les EC_{50} de la DA i la NE estan en el rang de submicromolar per a la majoria de proteïnes G i receptors i són relativament més altes que les obtingudes per AMPc. La DA és més potent que la NE per la majoria de receptors i proteïnes G, però es van trobar algunes diferències significatives. Per exemple, pels receptors D4R i D2R, la DA és 10 vegades més potent que la NE quan s'acobla a Gi1 però només 2 vegades quan s'acobla a Go1. D3R és el receptor amb més afinitat per la DA i les tres variants de D4R presenten una afinitat i eficàcia molt similar tant per la DA com per la NE.

Per comprovar que diferències en afinitat no siguin degudes a una expressió diferencial dels receptors, es van dur a terme experiments de FACS (*fluorescence activated cell sorter*) utilitzant un anticós Anti-Flag per mesurar el nivell d'expressió dels receptors a la membrana. L'anàlisi de FACS demostra que no hi ha diferències significatives en l'expressió de les isoformes D2SR i D2LR i les variants de D4R.

A continuació, es va analitzar la capacitat dels receptors D_2-like per reclutar arrestines quan són activats per DA i NE. Es va fer servir un assaig de BRET en el qual el receptor està fusionat a Rluc a la part C-terminal i la β-arrestina 2 està fusionada a Venus. Mitjançant aquest sistema es va comprovar que el rang de potència observat en els experiments anteriors es mantenia també en aquest assaig: D3R>D4R>D2; tant per DA com per NE.

Per últim, es van comparar els resultat dels receptors D_2-like amb els del receptor adrenèrgic α_{2A} (α2AR). Es va escollir aquest receptor ja que està també acoblat a proteïna G inhibitòria i es troba molt expressat al còrtex cerebral juntament amb D2R i D4R (Missale et al., 1998). Es van dur a terme experiments d'inhibició d'adenilat ciclasa, activació de proteïna Gαi1 i

reclutament de β-arrestina. Els valors d'EC_{50} de la NE per l'inhibició d'adenilat ciclasa i activació de proteïna Gαi1 es troben al voltant de nanomolar i són només uns 20 cops més baixos que per D3R i D4R. La potència de la NE per α2AR a l'assaig de reclutament d'arrestines és uns 4 cops més alta que pels receptors D_2-like.

DISCUSSIÓ

Fins ara, només el D4R s'havia descrit com un receptor "promiscu" que s'activa tant per DA com per NE i pot funcionar com un receptor adrenèrgic al cervell (Lanau et al., 1997; Newman-Tancredi et al., 1997; Czermak et al., 2006; Cummings et al., 2010; Root et al., 2015). Els nostres resultats indiquen que tot els receptors D_2-like poden unir i activar-se amb alta afinitat per NE i que les seves potències, especialment per D3R i D4R no són molt diferents de les del receptor adrenèrgic α2AR. L'aplicació de BRET de proteïna G ha permès mesurar l'activació de subtipus de proteïnes G específics i comparar l'efecte de les dues catecolamines. En general, sembla que la potència de la DA i NE depèn del subtipus de proteïna Gαi/o i del receptor.

Els valors de potència obtinguts amb els diferents assajos són en general consistents. A més, les variants de D4R no presenten diferències en cap dels assajos funcionals emprats mentre que les isoformes de D2R podrien tenir diferències en la seva capacitat d'acoblar-se a proteïnes G específiques.

Tenint en compte aquests resultats, el fenomen de reactivitat creuada (*cross reactivity*) entre DA i NE (Guiard et al., 2008; González et al., 2012a; Lei, 2014) podria ser explicat a nivell d'activació directa dels receptors i dels seus efectors i podria ser considerat com un fet biològic probable.

CONCLUSIONS

Per tant, les conclusions extretes d'aquest primer capítol són:

- La noradrenalina s'uneix i activa receptors D_2-like amb alta afinitat com s'ha demostrat amb experiments d'unió de radiolligands, inhibició d'adenilat ciclasa, activació de proteïna G i reclutament de β-arrestines.
- Les variants del receptor D_4 són funcionalment similars per tant podem hipotetitzar que les diferències es trobarien en la interacció amb altres receptors.
- L'aplicació de tècniques de BRET que permeten mesurar l'activació de subtipus de proteïnes Gi específics mostren una dependència de la proteína Gαi/o en la capacitat de la dopamina i la noradrenalina per activar els receptors D_2-like.

Capítol 2: Els receptors α_{2A}- i α_{2C} adrenèrgics són dianes de la dopamina i de lligands dopaminèrgics

INTRODUCCIÓ

La noradrenalina és un important neurotransmissor implicat en atenció, aprenentatge, memòria i estrés. S'uneix i activa tres famílies de GPCR: α_1 (α_{1A}, α_{1B}, α_{1D}), α_2 (α_{2A}, α_{2B}, α_{2C}) i β (β_1, β_2, β_3). El receptor α_{2A} (α2AR) és el subtipus més important en moltes zones cerebrals i està molt enriquit a l'estriat, juntament amb el receptor α_{2C} (α2CR). El receptor α_{2B} té una distribució més limitada i s'expressa preferentment al tàlem (Nicholas et al., 1993; Scheinin et al., 1994). Els baixos nivells de noradrenalina que es troben a l'estriat donen peu a la pregunta sobre quina és la funció dels receptors adrenèrgics (α2AR i α2CR) a l'estriat. S'ha descrit prèviament que la dopamina podria ser el neurotransmissor endogen dels receptors α_2 estriatals, tot i que amb resultats contradictoris, deixant la pregunta sense una resposta clara. Per aquestes raons es va formular el següent objectiu:

> **Objectiu 2:** estudiar l'activació de α2AR i α2CR per dopamina i lligands dopaminèrgics mitjançant estudis d'unió de radiolligands, activació de diferent subtipus de proteïna G i inhibició d'adenilat ciclasa.

RESULTATS

El mètode de BRET de proteïna G descrit anteriorment es va emprar per determinar la potència i l'eficàcia de la DA i altres lligands dopaminèrgics per activar els receptors α_{2A} (α2AR) i α_{2C} (α2CR) i comparar els diferents subtipus de proteïna Gi. A banda de DA i NE, en aquest estudi també es van incloure els següents lligands: l'agonista D2R-D3R-D4R quinpirole, l'agonista selectiu de D3R 7-OH-PIPAT i els agonistes selectius de D4R RO-105824 i PD-168077. Tant per α2AR com per α2CR, la DA mostra una gran potència i eficàcia comparat amb la NE. Tot i que la DA sempre és menys potent que la NE, la separació entre aquests dos lligands depèn de la proteïna G. La NE és més potent per α2AR que per α2CR, excepte per Gαi2 i Gαi3. La DA presenta una afinitat similar pels dos receptors, excepte per Gαi2 i Gαo1. En resum, utilitzant el mètode de BRET de G proteïna, podem concloure que la DA té alta afinitat per

α2AR i α2CR amb uns valors molt similars a les potències observades per alguns receptors D_2-like (capítol 1).

La clonidina (agonista α_2 selectiu) té més potència per α2AR que per α2CR activant les proteïnes Gαo1 i Gαo2. Una altra diferència important és que la clonidina es comporta com un agonista total de α2AR i com agonista parcial de α2CR, excepte per activar Gαi2 i Gαi3. Molt interessant és la variabilitat en el nivell d'eficàcia de la clonidina pel α2CR que passa d'una alta eficàcia sobre Gαi2 a una molt baixa eficàcia sobre Gαo1. L'agonista D3R 7-OH-PIPAT i l'agonista D2R-D3R-D4R quinpirole també activen α2AR i α2CR però amb perfils diferents. En general, 7-OH-PIPAT es comporta com un agonista total per α2CR i α2AR tot i que amb algunes excepcions. El quinpirole té poca afinitat per ambdós receptors (submicromolar) i funciona com un agonista parcial o total depenent de la proteïna G. L'especificitat del senyal produït pel quinpirole i el 7-OH-PIPAT es demostra utilitzant l'antagonista selectiu d'α_2 yohimbina. Finalment, els agonistes D4R selectius RO-105824 i PD-168077 no produeixen cap activació significativa dels receptors α2AR i α2CR. Sorprenentment, RO-105824 és capaç de competir amb l'agonista α_2 selectiu clonidina lo qual indicaria que RO-105824 podria actuar com antagonista/agonista parcial dels receptors α2AR i α2CR.

A continuació es van dur a terme experiments d'unió de radiolligands per tal de trobar una correlació amb els experiments d'activació de proteïna G. Es van fer assajos de competició entre l'antagonista d'α_2 adrenèrgic [^{3}H]RX821002 i els lligands emprats en l'apartat anterior, utilitzant preparacions de membranes de còrtex i estriat de xai. Els valors obtinguts es van ajustar al model dimèric "*two-state dimer model*" (Casadó et al., 2007) que considera els receptors com a dímers i no com a monòmers. Els valors de Bmax (número total de receptors) obtinguts demostren que la densitat dels receptors α_2 adrenèrgics és 3 vegades més gran al còrtex, el qual està majoritàriament representat per α2AR, mentre que a l'estriat s'expressen tant α2AR com α2CR.

Els resultats obtinguts per unió de radiolligands coincideixen en general amb els valors d'afinitat obtinguts en els experiments d'activació de proteïna G si considerem que els valors del còrtex representen α2AR mentre que els de l'estriat representen una combinació de α2AR i α2CR. En ambdós teixits NE, DA i clonidina presenten una alta afinitat amb un ordre de

potències de clonidina>NE>DA. L'afinitat de la NE és més gran al còrtex que a l'estriat mentre que l'afinitat de la DA és molt similar en ambdós teixits. Tots dos lligands presenten cooperativitat negativa en la seva unió al receptor adrenèrgic, la qual cosa vol dir que la unió de la molècula d'agonista al primer protòmer del homodímer de α2R disminueix l'afinitat del propi agonista per unir-se al segon protòmer. L'afinitat de la clonidina també és més alta al còrtex que a l'estriat, el que indica una major afinitat per α2AR que per α2CR. En resum, les altes afinitats de la NE i la clonidina al còrtex i les afinitats similars de la DA trobades als dos teixits correlacionen amb les diferències per α2AR i α2CR trobades en els experiments d'activació de proteïna G.

Els lligands sintètics 7-OH-PIPAT i quinpirole també desplacen la unió de [^{3}H]RX821002 amb afinitats de nanomolar i submicromolar, respectivament, lo qual correlaciona amb els experiments de BRET. Finalment, RO-105824 i PD-168077 mostren afinitats de subnanomolar i submicromolar, respectivament. Els experiments d'unió de radiolligands confirmen l'alta afinitat de l'agonista de D4R RO-105824 pels receptors α_2 adrenèrgics.

Els receptors α_2 adrenèrgics estan acoblats a proteïnes Gi i per tant la seva activació dóna lloc a la corresponent disminució d'AMPc. Estudis previs suggerien que els receptors α_2 podrien activar tant Gi com Gs (Fraser et al., 1989; Jones et al., 1991; Eason et al., 1992, 1994, 1995). Per tal d'estudiar l'efecte de la DA i la NE en l'inhibició de l'adenilat ciclasa, es va mesurar AMPc en cèl·lules transfectades amb α2AR i α2CR juntament amb CAMYEL. L'activació d'α2AR i α2CR amb DA i NE produeix a baixes concentracions, un efecte d'inhibició de la forskolina mitjançat per l'activació de Gi, i a altes concentracions, un efecte estimulador de l'adenilat ciclasa mitjançat per Gs. L'efecte inicial de disminució de l'AMPc proporciona valors d'EC_{50} que són qualitativament i quantitativa similars als observats en els BRETs d'activació de proteïna G on la NE era més potent que la DA. Cal remarcar que aquest efecte dual Gi/Gs pot ser bloquejat utilitzant l'antagonista selectiu d'α_2 yohimbina. Finalment, el tractament de les cèl·lules amb toxina *Pertussis* inhibeix l'efecte Gi, però manté l'aparent efecte mitjançat per Gs.

DISCUSSIÓ

Aquest estudi demostra que la DA s'uneix i activa α2AR i α2CR. La prova més concloent prové dels experiments de BRET d'activació de proteïna G, on les potències de la DA per α2AR i α2CR són similars o inclús més altes que per alguns subtipus de receptors D_2-like (Sánchez-Soto et al., 2016; Capítol 1). Per tant, podem hipotetitzar que irrespectivament de la concentració extracel·lular de NE a l'estriat, la concentració de DA hauria de ser prou alta com per activar α2AR i α2CR. La possibilitat d'una activació de α2AR i α2CR per DA a altres zones fora de l'estriat s'ha de tenir també en compte. Per exemple, el còrtex prefrontal rep certa quantitat de DA que podria unir-se i activar els receptors α2AR que es troben altament expressats.

El següent resultat obtingut d'aquest segon estudi és que α2AR i α2CR podrien ser també dianes de lligands prèviament caracteritzats com específics de D_2-like. Cal remarcar la capacitat dels agonistes selectius de D3R i D4R, 7-OH-PIPAT i RO-105824, respectivament, per unir-se i activar amb alta afinitat α2AR i α2CR. RO-105824 es comporta com un lligand molt potent per α2AR i α2CR amb una eficàcia intrínseca molt baixa, mentre que 7-OH-PIPAT es comporta de forma diferent depenent del receptor i el subtipus de proteïna Gi. De fet, tant la potència com l'eficàcia dels neurotransmissors endògens i dels lligands exògens semblen dependre tant del receptor com de la proteïna G. Aquest fet també es troba en l'estudi de receptors D_2-like descrits en el capítol anterior i sembla que es podria extendre a altres GPCRs i lligands. Tot i que les proteïnes G de la família Gαs-Gαolf mostren un patró d'expressió diferencial (Hervé et al., 2011), no tenim prou evidències per les proteïnes Gαi. Per tant, la caracterització del patró d'expressió dels diferents subtipus de proteïna Gαi és crucial per entendre la seva funció en l'activació de α2AR i α2CR així com l'estudi de lligands selectius de proteïna G.

CONCLUSIONS

Les conclusions extretes del segon capítol són:

- L'α2AR i α2CR són dianes importants per a la dopamina. Sorprenentment, les potències de la DA pels receptors α2AR i α2CR són molt similars o inclús més altes que per alguns subtipus de receptors D_2-like. Això pot tenir implicacions

importants per entendre la funció dels receptors α_2 a l'estriat i la seva possible activació per DA.

- L'α2AR i α2CR són també dianes per compostos prèviament descrits com agonistes dels receptors D_2-like. Particularment interessant és la capacitat dels agonistes de D3R i D4R, 7-OH-PIPAT i RO-105824 per unir i activar amb una alta afinitat els receptors α2AR i α2CR.
- Tant la potència com l'eficàcia dels lligands depèn del receptor i del subtipus de proteïna G tal i com s'ha demostrat també al capítol 1.
- La NE i la DA produeixen corbes dosi-resposta amb forma d'U-invertida tant per α2AR com per α2CR. Aquesta activació dual Gi/o-Gs depèn de la concentració de lligand: a concentracions baixes es produeix un efecte Gi mentre que a concentracions altes es produeix un efecte Gs.

Capítol 3: Els receptors D_4 de dopamina resultants de les dues variants polimòrfiques més comunes formen diferents complexes funcionals amb altres receptors de catecolamines

INTRODUCCIÓ

Actualment està acceptat que els receptors acoblats a proteïna G poden formar homo i heterooligòmers a la membrana plasmàtica. Aquestes interaccions entre proteïnes donen lloc a importants canvis farmacològics i funcionals dels receptors, per tant caracteritzar-les és important per entendre el seu rol al cervell i les seves implicacions terapèutiques. La funció específica del D4R al cervell no era del tot coneguda fins que experiments duts a terme al nostre laboratori *in vivo* han demostrat la funció del D4R en la transmissió cortico-estriatal. Cal destacar que la variant D4.7, que dóna lloc al D4.7R, produeix un guany de funció del receptor comparat amb el D4.4R que es considera el receptor D_4 *wild-type*. Evidències sortides del nostre i altres laboratoris suggereixen que la funció del D4R pot dependre de la seva interacció amb altres receptors ja que sembla que en cèl·lules transfectades el D4.7R té menys avidesa per establir interaccions intramoleculars i funcionals amb el receptor D_2 de dopamina (D2R) (Borroto-Escuela et al., 2011; González et al., 2012). De forma similar al D4R, el gen que codifica pel receptor α_{2A}-adrenèrgic (α2AR) conté molts polimorfismes que s'han associat amb el TDAH i la impulsivitat (Roman et al., 2003; Park et al., 2005; Stevenson et al., 2005). Degut a que tant D4R com α2AR estan involucrats epidemiològicament i farmacològica d'una forma similar en impulsivitat i la seva aparent promiscuïtat per ser activats per dopamina i noradrenalina, es va formular el tercer objectiu d'aquesta tesi:

> **Objectiu 3:** comparar la capacitat dels receptors $D_{4.4}$ i $D_{4.7}$ per formar complexes amb els receptors D_2 i $alfa_{2A}$ i activar proteïna G utilitzant diferents lligands que inclouen la dopamina i la noradrenalina.

RESULTATS

Primer vam voler estudiar l'efecte de lligands en diferents homo i heteròmers utilitzant la tècnica de CODA-RET (*Complemented Donor-Acceptor resonance energy transfer*). Aquest mètode biofísic permet l'estudi funcional de complexos formats per dos GPCRs definits i una subunitat de la proteïna G heterotrimèrica (Urizar et al., 2011). En aquest assaig, dos meitats complementàries de la Rluc es troben fusionades a cada un dels receptors mentre que la YFP es troba localitzada en una subunitat alfa de la proteïna G específica. Quan els dos receptors oligomeritzen la luciferasa pot reconstituir-se donant lloc a un donant funcional per la transferència d'energia cap a l'acceptor YFP. Utilitzant aquest mètode vam comparar l'activació de D4.4R-D2R amb D4.7R-D2R i vam analitzar l'efecte de DA, NE i també lligands dopaminèrgics (D_2-like) que s'empren actualment per al tractament de Restless leg syndrome (RLS) i la malaltia de Parkinson: pramipexole, ropinirole i rotigotina.

Els resultats obtinguts amb aquest mètode mostren com la NE i la DA activen D4.4R-D2R i D4.7-D2R amb potències similars a les de l'homodímer D2R-D2R. Comparant amb D2R-D2R, la potència de la DA en l'heteròmer D4.7R-D2R és major i la seva eficàcia relativa comparada amb la NE és més alta en l'heteròmer D4.4R-D2R. A continuació vam analitzar l'activació de proteïna G pels homòmers de D4R. En general, tant per D4.4R-D4.4R com per D4.7R-D4.7R la potència de la DA era major i la de la NE menor. Per tal de confirmar que els valors d'afinitat i eficàcia no eren producte d'una senyalització aberrant dels homodímers, es van fer experiments d'activació de proteïna G utilitzant receptors fusionats a Rluc (sense complementació). Similar als resultats anteriors, el D4.4R i D4.7R tenen una afinitat similar per la DA.

Finalment, per tal d'entendre quina era la contribució de cada receptor formant un heteròmer en l'activació de proteïna G per DA i NE, es va utilitzar l'antagonista selectiu de D2R-D3R raclopride i l'antagonista selectiu de D4R L745-870. Tant per DA com per NE, 100 nM de raclopride produeix una disminució quasi total en l'activació de D4.4R-D2R i D4.7R-D2R. En contra, L745-870 disminueix l'efecte de la DA i la NE en l'heteròmer D4.4R-D2R però no en el D4.7R-D2R.

A continuació es va analitzar l'efecte dels lligands dopaminèrgics en l'activació dels heteròmers D4R-D2R. Qualitativament, pramipexole, ropinirole i rotigotina es comporten de

manera similar en D4.7R-D2R i D2R-D2R: el pramipexole té alta eficàcia mentre que el ropinirole i la rotigotina són molt més parcials i amb un ordre de potència de rotigotina>pramipexole>ropinirole. Similar als experiments amb DA, els lligands dopaminèrgics tenen una major afinitat i eficàcia per D4.7R-D2R que D2R-D2R. Per altra banda, el pramipexole té major afinitat que la DA per l'heteròmer D4.4R-D2R, mentre que els altres dos lligands són totalment inefectius.

La tècnica de CODA-RET també es va utilitzar per estudiar la capacitat de D4R per interaccionar amb α2AR i comparar l'efecte dels diferents lligands endògens i exògens entre D4.4R-α2AR i D4.7R-α2AR. En l'homodímer α2AR-α2AR, la DA té una potencia similar a la de la NE, tot i que mostra menor eficàcia. L'eficàcia de la DA augmenta quan α2AR interacciona amb D4.4R o D4.7R; a més, l'afinitat de la DA incrementa en el dímer D4.4R-α2AR.

La contribució de cada receptor en els complexes D4.4R-α2AR i D4.7R-α2AR en la senyalització per DA i NE es va analitzar utilitzant l'antagonista de D4R L745-870 i l'antagonista selectiu de α_2 yohimbina. Molt similar als experiments anteriors amb D2R, la yohimbina produeix una inhibició casi total de l'activació, mentre que L745-870 només disminueix l'efecte de la DA i la NE en la parella D4.4R-α2AR.

Finalment es va analitzar la possible activació dels complexes D4.4R-α2AR i D4.7R-α2AR pels lligands D_2-like pramipexole, ropinirole i rotigotina i el seu efecte es va comparar amb l'homodímer α2AR-α2AR. Tant en el dimer D4.7R-α2AR com en l'homòmer α2AR-α2AR el pramipexole produeix una activació mínima (baixa eficàcia) mentre que ropinirole i rotigotina no produeixen cap efecte. Per altra banda, els tres lligands mostren una major afinitat amb una molt baixa eficàcia per D4.4R-α2AR.

DISCUSSIÓ

Aquest estudi indica que les variants de D4R no presenten diferències funcionals quan són activades per DA o NE. Això s'ha estudiat tant amb possibles "monòmers" com homodímers i va d'acord amb els experiments obtinguts en el capítol 1. Per altra banda, és l'associació de D4R amb d'altres receptors, D2R and α2AR, el que dóna lloc a diferències entre les variants de D4R.

Els lligands dopaminèrgics pramipexole, ropinirole i rotigotina són actualment teràpies pel tractament de RLS i del Parkinson. En aquest estudi es van utilitzar per dues raons, primer volíem comprovar si la promiscuïtat de la DA cap als receptors adrenèrgics era una propietat del neurotransmissor o també d'altres lligands dopaminèrgics. Segon, com s'ha dit anteriorment, els D4Rs, probablement formant heteròmers amb D2Rs controlen la transmissió glutamatèrgica cortico-estriatal i podrien ser dianes de la malaltia de RLS. Experiments de CODA-RET en heteròmers D4R-D2R i monòmers/homodímers D4R i D2 mostren que la rotigotina presenta diferències en la seva potència I eficàcia per les variants de D4R. A més, té una potència molt alta per l'heteròmer D4.7R-D2R, la qual cosa podria tenir implicacions funcionals.

Gràcies a l'ús del CODA-RET també hem pogut estudiar la capacitat de D4R d'interaccionar amb α2AR en cèl·lules transfectades. Similar als resultats anteriors, hem trobat algunes diferències entre els complexes D4.4R-α2AR i D4.7R-α2AR.

Les variants de D4R poden interaccionar amb D2R i α2AR però gràcies a l'ús d'antagonistes selectius per a cada receptor hem pogut descobrir que tot i físicament connectats, D4.7R sembla no ser functional. Aquests resultats es troben en la línia de treballs previs del nostre laboratori que suggerien que D4.7R no forma heteròmers funcionals amb D2R (González et al., 2012). Més experiments caldran en un futur per demostrar si aquest també és el cas del α2AR.

CONCLUSIONS

Les conclusions extretes del tercer capítol són:

- No hi ha diferències en la potència o l'eficàcia dels neurotransmissors endògens dopamina i noradrenalina en els monòmers o dímers D4.4R i D4.7R però l'associació d'aquests amb D2R or α2AR dóna lloc a diferències entre les dues variants de D4R.

- Per primer cop hem establert diferències en lligands exògens entre D4.4R i D4.7R. A més, l'associació amb D2R proporciona altres modulacions al·lostèriques en els heteròmers D4R-D2R.

- La rotigotina és un potent agonista de D4.7R-D2R comparat amb D4.4R-D2R or D2R-D2R. Això podria tenir implicacions pel tractament de RLS i de Parkinson.

- Hem demostrat l'existència d'interaccions intermoleculars i funcionals entre α2AR and D4R, els quals sembla que estan involucrats en la patofisiologia i el tractament de TDAH i addicció a drogues.

- El receptor α_{2A} forma complexes funcionals amb D4.4R però no amb D4.7R, aquest darrer associat amb el TDAH.