# Constitutive activity and drug functional selectivity of 5-$HT_{2A}$ receptors in post-mortem brain of subjects with schizophrenia


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## ABREVIATION LIST

| | |
|---|---|
| AA | Araquidonic acid |
| AC | Adenylyl cyclase |
| BB | Basal binding |
| $Ca^{2+}$ | Calcium ion |
| CaMKII | Calcium/calmodulin-dependent kinase II |
| CNS | Central nervous system |
| BRET | Bioluminiscence resonance energy transfer |
| cAMP | Cyclic adenosine monophosphate |
| DA | Dopamine |
| DAG | Diacylglycerol |
| DLPFC | Dorsolateral prefrontal cortex |
| (±)DOI | 2,5-dimetoxy-4-iodoamphetamine |
| DSM | Diagnostic and Statistical Manual of Mental Disorders |
| D2R | Dopamine 2 receptor |
| $EC_{50}$ | Concentration that promotes half-maximal stimulatory effect |
| $E_{max}$ | Maximal stimulatory effect |
| ERK | Extracellular signal-regulated kinases |
| FDA | Food and drug administration |
| GABA | γ-aminobutiric acid |
| GPCR | G-protein coupled receptor |
| GDP | Guanosine diphosphate |
| GRK | G-protein coupled kinase |
| GTP | Guanosine triphosphate |
| 5-HT | 5-Hydroxytryptamine (serotonin) |
| $5\text{-}HT_{2A}R$ | Serotonin 2A receptor |

| | |
|---|---|
| 5-$HT_{2A}R^{(-/-)}$ | Knock-out |
| 5-$HT_{2A}R^{(+/+)}$ | Wild-Type |
| GWAS | Genome-wide association studies |
| $IC_{50}$ | Concentration that promotes half-maximal inhibitory effect |
| $I_{max}$ | Maximal inhibitory effect |
| $IP_3$ | Inositol 1,4,5-tiphosphate |
| LSD | D-lysergic acid diethylamide |
| MAPK | Mitogen-activated-protein kinase |
| NBS | Non-specific binding |
| NMDA | N-methyl-D-aspartate |
| PCP | Phencyclidine |
| PET | Positron emission tomography |
| PFC | Prefrontal cortex |
| $PIP_2$ | Phosphatidylinositol 4,5-biphosphate |
| PKC | Protein kinase C |
| $PLA_2$ | Phospholipase $A_2$ |
| PLC | Phospholipase C |
| PMD | Post-mortem delay |
| PSD-95 | Postsynaptic protein density 95 |
| PTX | *Bordetella pertussis* toxin |
| RSK-2 | Ribosomal S6 kinase |
| GTPγS | 5'-O-[gamma-thio]triphosphate |
| [$^{35}$S]GTPγS | Sulphur 35-labelled guanosine-5´-O-(γ-thio)-triphosphate |
| SNP | Single nucleotide polimorphism |
| SPA | Scintillation proximity assay |
| 7TM | Seven transmembrane |
| Volinanserin | MDL100907 |

# INDEX

# Introduction

## 1.1 Schizophrenia

### 1.1.1 Definition

Schizophrenia is a chronic and disabling mental disorder characterized by heterogeneous behavioural and cognitive syndrome, which includes hallucinations, anhedonia and cognitive impairments (Owen et al., 2016; Jauhar et al., 2022). Lifetime prevalence of schizophrenia is estimated between 0.3-0.7% of worldwide population (McGrath et al., 2008). Schizophrenia development presents a higher frequency in men than in women (Jongsma et al., 2019) and typically develops the first episode of psychosis in late adolescence or early adulthood. Furthermore, the average age of onset is in the late teens to early twenties for men (Kirkbride et al., 2012), and the late twenties to early thirties for women (Ochoa et al., 2012).

Schizophrenia is regarded as one of the most severe and disabling psychiatric disorders, which can shorten life expectancy by 10-20 years (Chesney et al., 2014). Moreover, suicide is a common cause of death in patients with schizophrenia; showing a 5% of life risk (Hor & Taylor, 2010).

### 1.1.2 Symptomatology

The diagnosis of schizophrenia is made clinically based on history and examination of the mental state. Right now, no diagnostic test or biomarkers are available and, therefore, the clinical diagnosis of schizophrenia is based on criteria of Diagnostic and Statistical Manual of Mental Disorders (DSM) from American Psychiatric Association, or on International Statistical Classification of Diseases and Related Health Problems from Word Health Organization (Kendler, 2016).

Schizophrenia is characterized by a diverse psychopathology. Hence, clinical manifestations are grouped in three main categories: positive, negative and

cognitive symptoms. Positive symptoms are the core features of schizophrenia, and include hallucinations (most frequently auditory), disillusions and psychotic behaviour, in which contact with reality is lost. Positive symptoms tend to appear in episodes, and usually spaced in time, although some patients have residual long-term psychotic symptoms. Negative symptoms are characterized by impaired motivation, reduction in spontaneous speech and social withdrawal (Liddle, 1987). Finally, cognitive symptoms include difficulties in attention and concentration, learning, memory and executive functions (Joyce & Roiser, 2007).

### 1.1.3 Aetiology of schizophrenia

Schizophrenia is a complex disease probably caused by multiple aetiological factors. Thus, genetic and environmental factors are known to take part in the onset and development of schizophrenia (Sullivan et al., 2012; McCutcheon et al., 2020). Moreover, a neurodevelopmental aetiological hypothesis has been proposed and associated with structural, functional and neurochemical brain changes. Those changes affect several neurotransmission systems and circuits that seem to be affected in patients with schizophrenia. Evidence points to abnormalities in dopamine (DA), serotonin (5-HT) and glutamate neurotransmitter systems in the pathology of schizophrenia. Even so, none of the hypothesis seems to be sufficient to explain the full spectrum of the disease.

### 1.1.3.1 Genetics

Many epidemiological studies have consistently shown a genetic component in the development of schizophrenia, with an estimated heritability nearly 80% (Sullivan et al., 2003). In recent years, several large-scale genomic studies have allow to study the contribution of specific deoxyribonucleic acid variants and different type of risk alleles. In consequence, schizophrenia is currently considered a highly polygenic disorder.

Genome-wide association studies (GWAS) have shown that multiple common variants are associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomic Consortium, 2014). Some of the most replicated gene association involves postsynaptic density (PSD) proteins, activity-regulated cytoskeleton-associated protein, N-methyl-D-aspartate (NMDA) receptor, fragile X mental retardation protein targets as well as other neurodevelopment disorders, voltage-gated calcium ($Ca^{2+}$) channels, neural cell adhesion molecule and dopamine 2 receptor ($D_2R$) (Pardiñas et al., 2018; Trubetskoy et al., 2022). A widely accepted association of genes with schizophrenia arises in part from many structurally diverse alleles from complement component 4 genes that is related with the histocompatibility complex (Sekar et al., 2016).

### 1.1.3.2 Environmental factors

Environmental factors are also involved in the aetiology of schizophrenia. Although studies indicated a strong genetic influence and high heritability, it has been proven that different environmental factors can trigger the disease in people who already have a genetic predisposition, a term known as “stress-vulnerability” model (Os et al., 2010).

The environmental factors have been related with the neurodevelopmental hypothesis of schizophrenia (Fatemi & Folsom, 2009). Numerous studies have consistently reported increased incidence of schizophrenia associated to

several factors that affect early neurodevelopment during pregnancy, including maternal stress, maternal infections, nutritional deficits as well as birth complications (Jones et al., 1998; McGrath et al., 2010; Brown, 2012) (**Figure 1.1**). Several studies have revealed increased risk for developing psychosis associated to childhood adversities (Varese et al., 2012). Furthermore, well stablished evidences show that socioeconomic factors (Allardyce & Boydell, 2006) and immigration (both first and second generations) show relationship with rates of schizophrenia (Cantor-Graae & Selten, 2005). Toxic conditions also play a role in schizophrenia. Thus, accumulating evidence pointed to association between cannabis use and psychosis (**Figure 1.1**). These studies suggested that early chronic exposure to cannabis is associated with a higher vulnerability for psychotic outcomes, including later schizophrenia development (Moore et al., 2007; Ibarra-Lecue et al., 2018) (**Figure 1.1**). Therefore, many candidate genes and environmental risk factors seem to be robustly associated with schizophrenia.

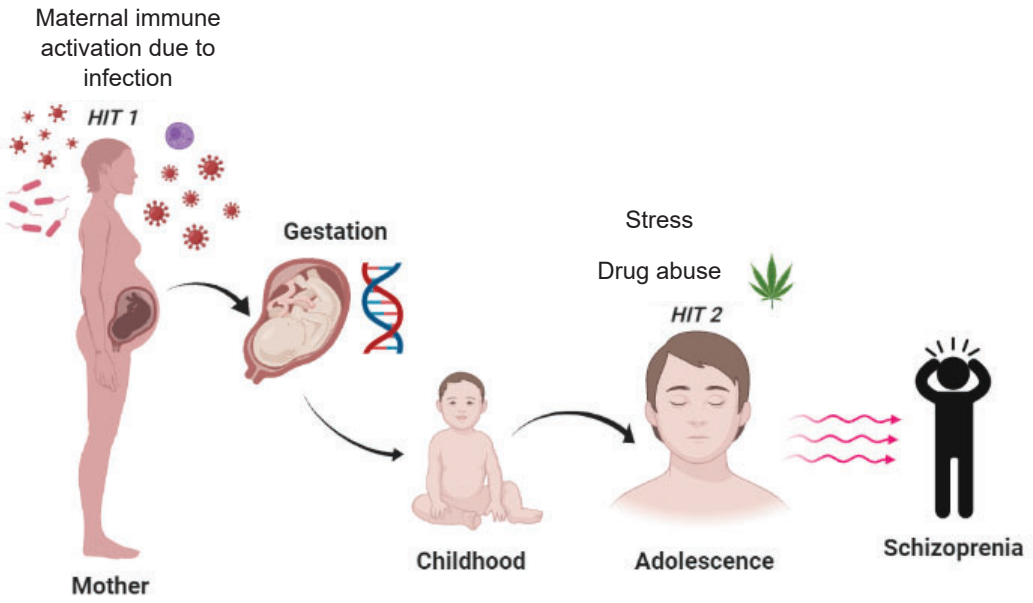



**Figure 1.1**: Schematic representation of how environmental factors can lead to psychiatric disorders, such as schizophrenia in offspring. Infection during pregnancy induces pro-inflammatory cytokines release and immune system activation. Genetic background, autoimmune status, and second hits during childhood and adolescence (including stress and drug abuse) combined with consequences of maternal infection increase the likelihood of offspring to develop psychiatric disorders in adulthood. *Illustration originally created for this thesis by N Cordero and adapted from Estes & McAllister, 2016*.

### 1.1.3.3 Neurotransmission systems alterations in schizophrenia

#### Dopaminergic hypothesis of schizophrenia

The most widely known theory to explain clinical symptoms and drug response of schizophrenia is the dopaminergic hypothesis, which is based on different findings. First, clinical effects of typical or first-generation antipsychotic are related with their ability to block $D_2R$ (Seeman & Lee, 1975). Second, drugs that increase DA activity, such as amphetamine, produce psychotic episodes in healthy individuals, and worsened psychosis in schizophrenic patients (Lieberman et al., 1987).

Dopaminergic hypothesis argues hyperactivity of dopaminergic transmission in mesolimbic and striatal regions that would be responsible of positive symptoms (hallucinations, delusions). However, negative and cognitive symptoms shown to be resistant to antipsychotics. Thus, dopaminergic hypothesis was reformulated postulating that a prefrontal hypodopaminergia, more than hyperdopaminergia, would contribute to negative and cognitive symptoms (Davis et al., 1991; Howes & Kapur, 2009; McCutcheon et al., 2020).

Dopamine receptors are G-protein coupled receptors (GPCR) that can be divided into two main types: dopamine $D_1$ receptor family, including $D_1$ and $D_5$ dopamine receptors ($D_1R$, $D_5R$), and $D_2$ receptor family, including $D_2$, $D_3$ and $D_4$ dopamine receptors ($D_2R$, $D_3R$, $D_4R$). The $D_2R$ is a main target of typical and atypical antipsychotic medication, which suggest that this receptor plays a key role in schizophrenia.

*In vivo* neuroimaging, based on positron emission tomography (PET) and single photon emission computerized tomography techniques (SPECT), have been used to evaluate the status of $D_2R$ and $D_1R$ in schizophrenia patients. Initial studies in basal ganglia were inconsistent, with some of them reporting increased $D_2R$ density and others no differences from controls (Howes et al., 2012; Cumming et al., 2021). Elevated $D_2R$ density was suggested to be a consequence of receptor up-regulation after long-term antipsychotic medication, since drug-naïve patients did not show such PET alterations (Seeman, 2013). In *vitro* post-mortem binding studies corroborated the finding of enhanced striatal $D_2R$-binding density in schizophrenia (Seeman et al., 1984; Zakzanis & Hansen, 1998). Moreover, a recent study has revealed an absence of striatal $D_2R$ hyperactivity in post-mortem brain of schizophrenia subjects (Egusquiza et al., 2021). As for $D_2R$, *in vivo* observations on $D_1R$ density in prefrontal cortex (PFC) of drug-naïve schizophrenia patients have shown discrepancies (Cumming et al., 2021). In contrast to this earlier focus

on postsynaptic $D_1R$ and $D_2R$ dysregulation, more recent findings point towards a critical role of presynaptic dopaminergic neurotransmission system in schizophrenia. Thus, elevated presynaptic DA synthesis capacity (Howes et al., 2012; Fusar-Poli & Meyer-Lindenberg, 2013), higher synaptic DA concentration (Abi-Dargham et al., 2000; Caravaggio et al., 2015), and amphetamine-induced DA release (Howes et al., 2012; Laruelle, 1998) have been demonstrated in striatum of schizophrenia subjects. Conversely, evidence suggest a reduction of amphetamine-induce DA release in frontal cortex of schizophrenia patients (Slifstein et al., 2015), indicating a presynaptic hypodopaminergia in this brain area (Slifstein et al., 2015).

## Glutamatergic hypothesis of schizophrenia

Glutamate is the major excitatory neurotransmitter in the brain. Glutamate interacts with selective ionotropic and metabotropic receptors. Ionotropic receptor group includes NMDA, kainate and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptor subtypes. Metabotropic glutamate receptors (mGluRs), which activate G-protein signal transduction, are divided in groups I ($mGlu_1$, $mGlu_5$), II ($mGlu_2$, $mGlu_3$) and III ($mGlu_6$, $mGlu_4$, $mGlu_7$, $mGlu_8$) (Nakanishi, 1992; Niswender & Conn, 2010; Muguruza et al., 2016).

It is well stablished that administration of NMDA receptor antagonists, such as phencyclidine (PCP) or ketamine, can induce psychosis-like states and cognitive deficits in healthy humans. Ketamine and PCP are non-competitive glutamate NMDA antagonist (Thomson et al., 1985). This fact gave support to the hypothesis of a functional impairment of NMDA receptors in schizophrenia (Javitt & Zukin, 1991; Stone et al., 2008). Interestingly, addition of amphetamine or other dopaminergic agonist as well as NMDA receptor antagonist also reassembles psychotic symptoms, and evokes cognitive and negative symptoms, better mimicking the pathophysiolology of schizophrenia

(Krystal et al., 2005). For this reason, non-competitive NMDA receptor antagonist administration has been chosen as a usual animal model of schizophrenia.

Multiple reports have evaluated the status of glutamate in brain of schizophrenia patients and its relationship with different spectrum of symptoms. Thus, evidence that impaired glutamatergic system might be implicated in the cognitive dysfunction in schizophrenia is currently acknowledged (Moghaddam & Javitt, 2012).

Glutamate hypothesis is indeed closely related to hyper- and hypoactivity of dopaminergic pathways in limbic and cortical areas, respectively. First, it has been described an hypoactivity or inhibition of NMDA receptors located in γ-aminobutiric acid (GABA)ergic interneurons in mesolimbic dopaminergic areas. This would impair the inhibitory tonic state, resulting in higher synthesis and release of dopamine in those areas, which could predispose to psychotic symptoms. In contrast, cognitive and negative symptoms could be related with a hypofunctionality of NMDA receptors in the cortex-brainstem projection, which could make hypoactive mesocortical dopaminergic pathways (Javiit, 2010).

## Serotonergic hypothesis of schizophrenia

The 5-HT hypothesis of schizophrenia arose from early studies on interactions between the hallucinogenic drug D-lysergic acid diethylamide (LSD) and 5-HT (Freedman 1961). LSD and related compounds produce mental disturbances, resembling those occurring at the onset of schizophrenia, and mediate their central effects through 5-HT receptors. Moreover, psychedelic drugs have some chemical similarities with 5-HT structure.

Among all the 5-HT receptor subtypes, abundant evidence indicate that the effects of hallucinogens are mediated through the serotonin 2A receptor (5-

$HT_{2A}R$) subtype (González-Maeso et al., 2007; Geyer & Vollenweider, 2008; González-Maeso & Sealfon, 2009; Madsen et al., 2019). In relation with these facts, atypical antipsychotic drugs show high affinity for 5-$HT_{2A}R$, making this target one of the most widely studied in schizophrenia (Meltzer et al., 1989). A more detailed review of 5-$HT_{2A}R$ physiology and involvement in schizophrenia can be found in **section 1.4**.

### 1.1.4 Morphological brain alterations

PFC has long been implicated in the pathophysiology of schizophrenia, especially in negative and cognitive manifestations (Kolk & Rakic, 2022). In fact, lesions of this brain region in animals and human might produce latter impairments, as disruption in working memory, reduced impulsive choice, enhanced environmental stimuli and behavioural restrains. Thus, abnormalities in PFC have been largely associated with schizophrenia and other psychiatric diseases (Xu et al., 2019).

It is well described that schizophrenia patients exhibit lateral ventricular enlargement by 25% of volume, which is accompanied by a whole brain volume reduction around 2% (Johnstone et al., 1976; Haijma et al., 2013). The ventricles size increases progressively after the onset of the illness, as whole brain grey matter is reduced over time (van Erp et al., 2016).

Morphological studies in post-mortem brains of schizophrenia patients have revealed differences in cellular distribution, likely attributed to altered neuronal migration early in brain development, loss of pyramidal cells, malformed cell structure, and decreased number of GABA interneurons (Schmidt & Mirnics, 2015).

## 1.2 G-protein coupled receptors (GPCRs)

### 1.2.1 General aspects

GPCRs, also known as seven transmembrane (7TM) receptors, form one of the largest membrane receptor subfamily of plasma membrane protein in the body. These receptors bind to diverse type of ligands, such as ions, small molecules and peptides, linking them to downstream signalling (Alexander et al., 2019).

GPCRs have an enormous biomedical relevance because they are involved in diverse physiological activities, and play a crucial role in pathogenesis of disorders, being important as drug-targets. Moreover, it is estimated that approximately 35% of approved drugs target GPCRs (Hauser et al., 2017).

The 7TM receptor superfamily is also termed as GPCRs because exerts effects in response to different ligands through the association with G-proteins. G-proteins are heterotrimeric guanine nucleotide binding proteins, constituted by three subunits: α, β and γ. However, GPCRs can also bind to other cytosolic adaptors, including β-arrestins, which elicit G-protein independent activation (Syrovatkina et al., 2016). G-proteins mediate the earliest step in cell response to external events, by linking cell surface receptors to intracellular signalling (Sriram & Insel, 2018). G-proteins serve as transducers or amplifiers of signals from GPCRs to intracellular effectors.

### 1.2.2 The basic mechanism of GPCR signalling

Heterotrimeric G-proteins have a crucial role in defining the specificity and temporal characteristics of cellular responses. Upon ligand activation, there is a conformational change of GPCRs that increase their affinity for G-proteins, leading to G-protein recruitment. The interaction of a G-protein with an active receptor stimulates the exchange of guanosine diphosphate (GDP) nucleotide

(inactive conformation) for guanosine triphosphate (GTP) (active conformation) (**Figure 1.2**). In the inactive conformation, Gα subunit is bound to GDP as well as to Gβ and Gγ subunits forming a heterodimer. After receptor activation, Gα subunit bounds to GTP and dissociates from the Gβγ dimer (Gilman, 1987; Marinissen & Gutkind, 2001). Afterwards, Gβγ subunits modulate downstream cellular signalling pathways, such as adenylyl cyclase (AC), phospholipase and ion channels (Hamm, 1998). The signal terminates when the GTP is hydrolysed by the GTPase activity of Gα subunits, to GDP and phosphate, and the Gβγ complex binds to Gα, forming the inactive G-protein (Milligan & Kostenis, 2006; Hilger et al., 2018) (**Figure 1.2**).

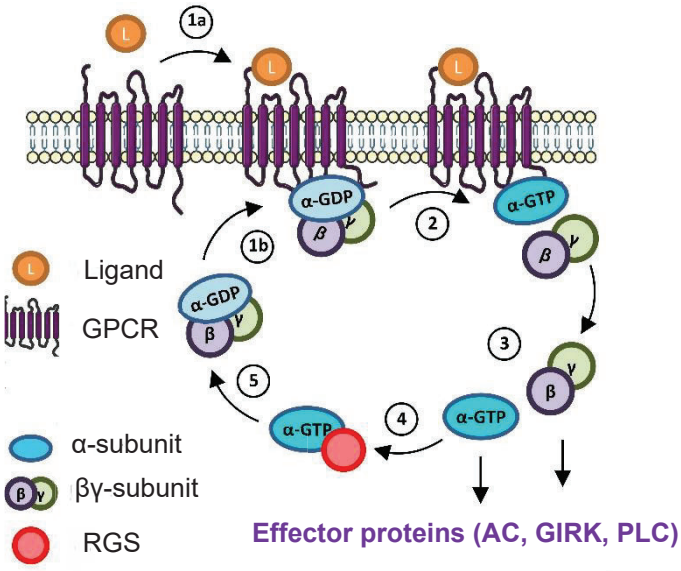



**Figure 1.2**: Schematic representation of GPCR/G-protein activation model. In the resting or inactive state G-proteins are heterotrimers comprising the α-subunit (bound to GDP) and βγ-subunit (1a). On binding of a ligand (as a neurotransmitter), GPCRs undergo conformational change that permits G-protein binding (1b) and catalyses the exchange of GDP for GTP on the Gα-subunit and the separation of βγ subunit (2). At this point, G-protein is active allowing α and βγ subunit interact with downstream effectors to modulate different signalling pathway (3). The signal is terminated on hydrolysis of GTP to GDP by Gα, which may be catalysed by the binding of Regulators of G-proteins Signalling (RGS) to the Gα-subunit (4). Finally, GTP hydrolysis results in re-formation of the trimeric complex, binding the G-protein back to its inactive state (5).(AC= Adenylyl cyclase, GIRK= G-protein-sensitive inwardly rectifying $K^+$ channel, PLC= Phospholipase C). *Illustration adapted from de Oliveira et al., 2019.*

The agonist binding to GPCRs not only initiates conformational changes that stimulate G-proteins, but also facilitates receptor phosphorylation by G-protein coupled kinases (GRKs). This phosphorylation promotes the recruitment of β-arrestins, which are critical steps for GPCR down-regulation and attenuation of G-protein-dependent signalling. Moreover, β-arrestins are able to elicit G-protein independent signalling, through activation of the mitogen-activated-protein kinase (MAPK) pathway (Wang et al., 2018).

Apart from binding to G-proteins and β-arrestins, GPCRs can also interact with similar or other GPCRs to form dimers, as well as higher order oligomers that are often essential for modulation of GPCR function (Milligan et al., 2019).

## G-proteins

G-protein heterotrimers are typically grouped into four main classes, based on their constituent Gα subunit: $G\alpha_{s/olf}$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Simon et al., 1991). After receptor activation, each Gα-protein family activates distinct signalling pathways, resulting in diverse physiological responses (**Figure 1.3**).

$G\alpha_s$ family has two members, $G\alpha_s$-protein, which is expressed in most cell types, and $G\alpha_{olf}$-protein, which is mainly expressed in the olfactory sensory neurons (Weinstein et al., 2007). $G\alpha_s$ family stimulates AC, stimulating their catalytic activity and, thus, stimulating cyclic adenosine monophosphate (cAMP) production (Milligan & Kostenis, 2006).

In contrast, $G\alpha_{i/o}$ family inhibits AC, resulting in reduction of cAMP (Busnelli et al., 2013). This G-protein family includes $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{i3}$-, $G\alpha_o$- and $G\alpha_z$-protein subtypes. $G\alpha_i$ subunits are found in most cell types, including the brain, and share 85-90% of homology between them (Plummer et al., 2012). $G\alpha_o$ is highly expressed in neurons, and it corresponds to the most abundant G-protein in the brain (Sternweis & Robishaw, 1984). In the same way, $G\alpha_z$ expression is found in neuronal tissue and platelets (Hultman et al., 2014). Moreover, all members of this family (with exception of $G\alpha_z$) contain a conserved cysteine residue on the carboxyl terminus domain. This corresponds to the site of adenosine diphosphate (ADP)-ribosylation, catalysed by *Bordetella pertussis* toxin (PTX) that results in the blockade of Gα-protein activation (Morris & Malbon, 1999). For this reason, this family is considered as PTX-sensitive G-proteins.

Members of $G\alpha_{q/11}$ family activate phospholipase β (PLC-β), leading to the synthesis of inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG) from the membrane phospholipid phosphatidylinositol 4,5-biphosphate ($PIP_2$). $IP_3$ mobilizes calcium from endoplasmatic reticulum, while DAG activates protein kinase C (PKC) (Wettschureck et al., 2005; Wilkie et al., 2021). $G\alpha_{q/11}$ family consist of $G\alpha_q$-, $G\alpha_{11}$-, $G\alpha_{14}$- and $G\alpha_{15/16}$-proteins. $G\alpha_{11}$- and $G\alpha_q$-proteins are ubiquitously expressed, and share 88% of aminoacid sequences (Wilkie et al., 1991). In contrast, $G\alpha_{14}$- and $G\alpha_{15/16}$-protein expression is more limited to other tissues, such as kidney (Tanaka et al., 2000).

Finally, the $G\alpha_{12/13}$-protein family stimulates Rho guanine nucleotide exchange factors (RhoGEFs), and it has two subfamilies, $G\alpha_{12}$- and $G\alpha_{13}$-proteins, which are expressed in most type of cells (Siehler, 2009).

Even if originally it was thought that GPCRs could only couple to a single G-protein, it is now established that GPCRs are promiscuous, and they can simultaneously signal via different G-protein families, generating different signalling cascades.

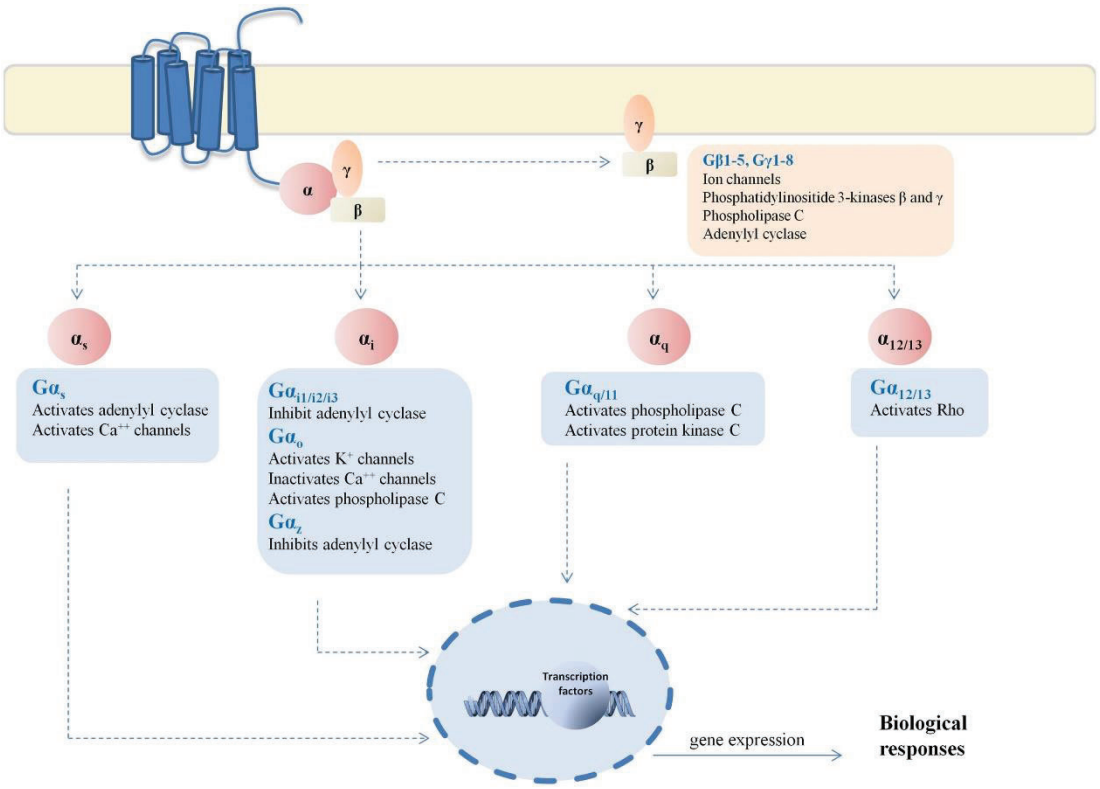



**Figure 1.3**: Representative scheme of signalling pathways linked to each G-protein. *Illustration from Diez-Alarcia et al., 2016.*

### 1.2.3 Theory of drug receptor interaction for GPCR

The action of drugs on GPCRs depend on two fundamental events. Firstly, the ligand must bind to the receptor, which may depend on the drug affinity for that receptor. Secondly, the ligand may have effects on the receptor inducing a conformational change, which is associated to a signalling system, and that has been termed as efficacy (Kenakin, 2002).

Drug affinity is defined as the compound ability to bind a receptor that is unique for each drug-receptor pair. Furthermore, numerically, affinity is the reciprocal of the equilibrium dissociations constant ($1/K_D$), which represents the concentration of a drug needed to occupy 50% of receptor population (Strange, 2008). Potency of a drug is the functional value that informs about

its affinity. The affinity of each ligand for each receptor might define the selectivity, which limits the therapeutic effect of a drug. Generally, drugs display several adverse effects together with therapeutic efficacy. These adverse reactions are commonly due to the lack of selectivity for a single receptor (Roth et al., 2004).

Efficacy depends on cellular context. The translation of GPCR affinity to cell signalling is influenced by transducers, such as G-proteins. Ability to induce responses or efficacy is also referred as intrinsic efficacy. This is the property of a drug that defines the strength of the receptor stimulus, which is ultimately converted into a response by the cellular signalling machinery (Kenakin, 1999). The cellular response to a specified concentration of a drug is composed of both system-dependent properties (receptor density and efficiency of receptor-effector coupling) and system-independent, drug-dependent properties (affinity and intrinsic efficacy) (Berg & Clarke, 2018). Specific potency and efficacy of each can be measured using concentration dose-response curves (**Figure 1.4**). Concentration that promotes half-maximal effect ($IC_{50}$/$EC_{50}$), as well as maximal inhibitory/stimulatory effect ($I_{max}$/$E_{max}$) values can be calculated, and the values provide the information about the drug potency and efficacy (Strange, 2008; Wacker et al., 2017). Based on efficacy, ligands were initially classified according to their relative responses respect to endogenous ligands in full agonists, partial agonists and antagonists. Later, the concept of inverse agonist emerged to explain unexpected functional responses of theoretical antagonists that did not behaved as neutral antagonists.

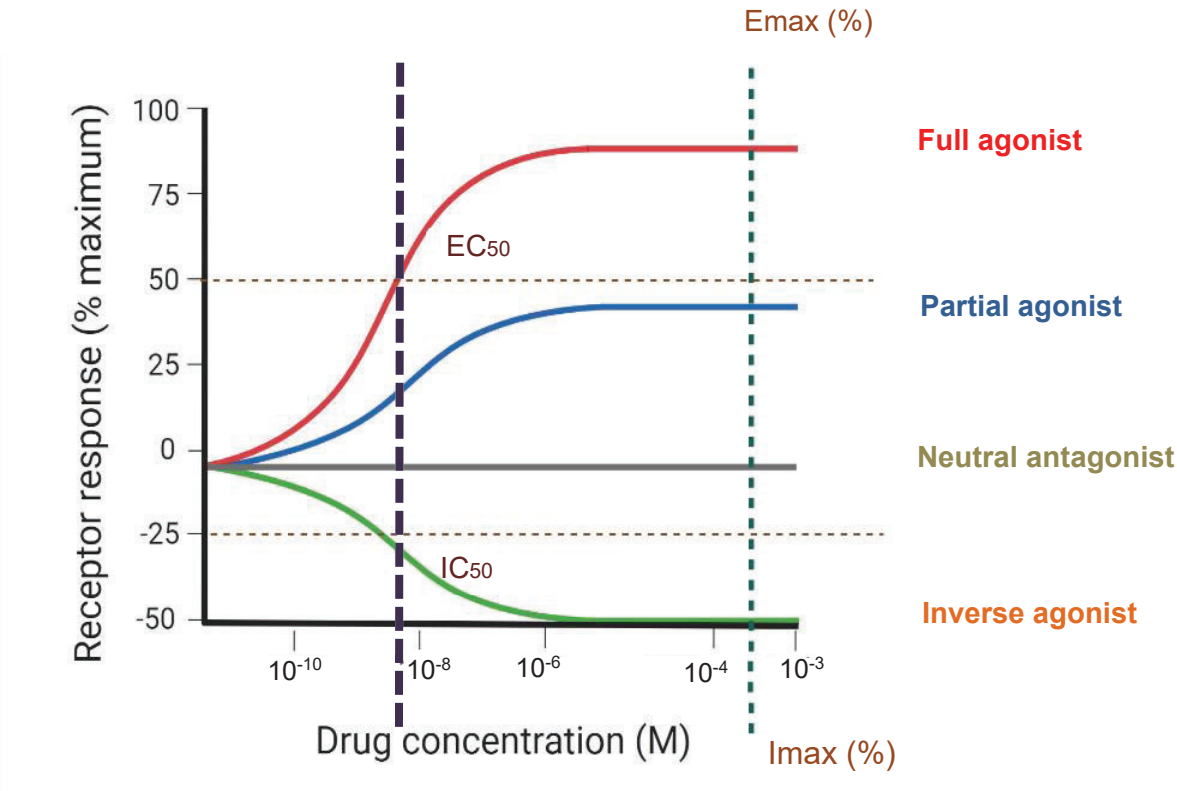



**Figure 1.4**: Schematic representation of concentration-response curve for GPCRs. On the Y axis, percentage defines the receptor response, being the 100% the maximal response and 0% lack of response. X axis shows the increasing concentrations of a ligand. 0% corresponds to constitutive or basal activity of the receptor in absence of any ligand. Thus, **full agonists** exert the maximal response, while **partial agonists** do not reach maximal response. **Neutral antagonists** present lack of intrinsic efficacy and do not modify the constitutive activity. In contrast, **inverse agonists** decrease or inhibit basal constitutive activity. Maximal stimulatory or inhibitory response ($I_{max}/E_{max}$) corresponds to the maximal effect of a ligand. Concentration needed to reach half-maximal response of each ligand corresponds respectively to $EC_{50}$ or $IC_{50}$ value.

Agonists present affinity for a target receptor, as well as efficacy. Thus, these ligands are able to bind the receptor and subsequently produce a response. Agonists can differ in their magnitude of the receptor-produced stimulus, which leads to their characterization as full or partial agonist. A full agonist produces maximal effect, while a partial agonist exerts a submaximal response, depending on their intrinsic efficacy. In contrast, antagonists display affinity but not intrinsic efficacy, since they do not induce conformational changes in the receptor to promote response. Its impact depends on their competence to reduce the probability of an agonist, such as endogenous ligand, to bind the receptor and decreasing endogenous cellular response.

On the other side, Samama et al. proposed that the presence of a ligand is not necessary to generate a cellular response or signalling (Samama et al., 1993), which is defined as constitutive activity. Thus, the constitutive activity consists on spontaneous auto-activation of the receptor, adopting a conformation able to trigger intracellular signalling despite ligand absence (Lefkowitz et al., 1993). In this sense, Costa and Herz found that ligands with negative intrinsic efficacy were able to decrease the constitutive activity (Costa & Herz, 1989; Chidiac et al., 1994; Costa & Cotecchia, 2005). These, ligands were named as inverse agonists, and could be classified as full or partial inverse agonists, according to their intrinsic efficacy.

Ternary complex model is the most widely accepted GPCR signalling model (Lean et al., 1980). According to this model, receptor is in a dynamic equilibrium between inactive (R) and active (R*) conformational states. Based upon this model, neutral antagonists have identical affinities for inactive and active conformational states, whereas agonists exhibit higher affinity for the active state. Because agonists have higher affinity for the active conformation of the receptor, agonist binding stabilizes GPCR in its active state, shifting the dynamic equilibrium from R to R*.

The maximal effect of an agonist (efficacy) is directly dependent upon the differential affinity of ligand for inactive receptor conformation (R) versus active receptor conformation ($R^*$). Conversely, inverse agonists exhibit higher affinity for inactive receptor state (R). Therefore, inverse agonist binding results in the stabilization of the inactive state (R), shifting the dynamic equilibrium from R* to R. However, the inverse agonist efficacy is also dependent upon the magnitude of constitutive activity (**Figure 1.5**).

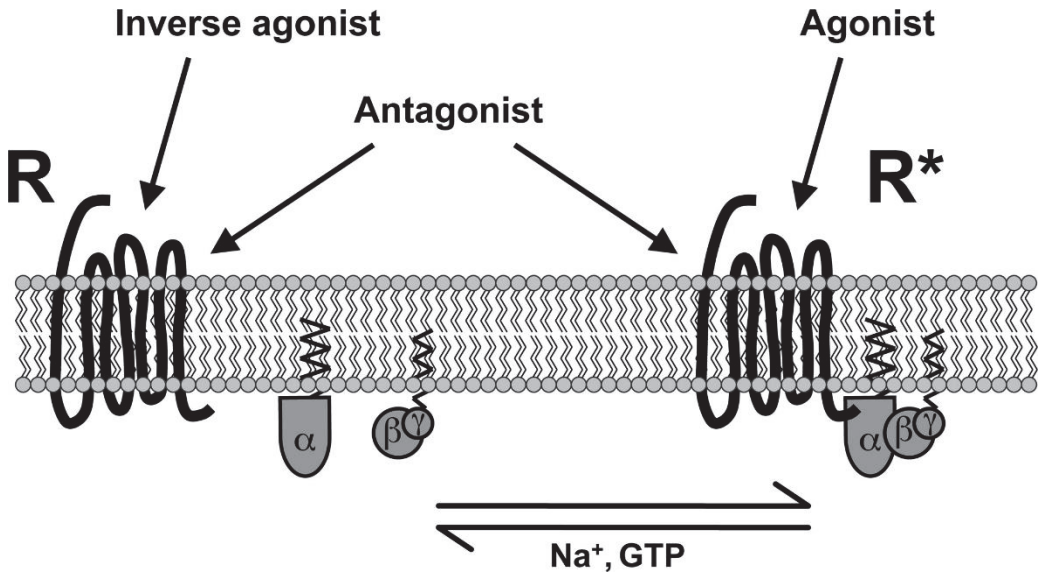



**Figure 1.5**: Schematic representation of agonist, antagonist and inverse agonist binding to different functional states of the GPCR. Agonist binds with high affinity to active conformation of receptor (R*), whereas inverse agonist preferentially binds and stabilizes receptor in the inactive state (R). Neutral antagonist binds with the same affinity both active and inactive states of GPCR. *Illustration from Muguruza et al., 2013.*

According to the extended ternary complex model, a receptor is able to switch from an active to inactive state in absence of a ligand (Sammama et al., 1993). In this sense, inverse agonism is considered the pharmacological property of a drug to antagonize the action of agonists, and simultaneously decrease basal constitutive activity of receptor signalling. Consequently, demonstration of inverse agonist properties requires the existence of constitutive basal

activity that will be reduced by the inverse agonist. The decrease of constitutive activity must be sensitive to blockade with an antagonist, and must be absent in knock-out animals for the involved receptor (Aloyo et al., 2009). After inverse agonism scientific description, many antagonist originally thought to be “neutral” turned out to be inverse agonist ligands (Strange, 2002; Kenakin, 2004).

Although these findings indicate a potential usefulness of inverse agonists, there is little information about the use of pharmacological properties of these drugs in therapeutics. For example, somatic receptor mutations leading to constitutive active receptors are a causal factor in certain diseases, such as male precocious puberty (Kosugi & Mori, 1995). Thus, inverse agonists might be beneficial here, since they would decrease receptors basal activity induced by the mutation. In this case, neutral antagonists would presumably be of little use, in absence of increased endogenous ligand levelstt (Ligt et al., 2000). Overall, the concept of inverse agonist might provide new opportunities in such screening strategies. For that purpose, functional assay describing constitutive activity are need. These assays could contribute to the identification of agonist as well as inverse agonist ligands.

### 1.2.4 GPCRs and biased signalling

As mentioned above, GPCRs can signal simultaneously through parallel pathways such as heterotrimeric G-proteins, β-arrestins and GRKs. In this context, several ligands have been described to differ in their ability to engage the different signalling pathways coupled to the respective GPCR, a term called biased signalling or functional selectivity (Azzi et al., 2003; Galandrin et al., 2007; Perez & Karnik, 2005; Kenakin, 2011; Kenakin, 2012; Smith et al., 2018). Biased agonists are theoretically able to stabilize different receptor conformations, exhibiting different affinities for the multiple signal transducers of the GPCR. They produce, as consequence, different cellular responses. In

contrast, other ligands can equally activate all signalling pathways. These drugs are termed balanced agonists or un-biased ligands.

Interestingly, activating or inhibiting specific signalling cascades represents a new approach to effect selectivity, which could yield to improved therapeutically effective drugs, with lower side effects. In this way, the search for ligands for a particular signalling pathway, rather than selective for specific receptors, is one of the main challenges of drug development nowadays (Komatsu et al., 2019).

The best well-known example of biased signalling is present in opioids pharmacology (Che et al., 2021). Biased signalling involves a differential activation between Gαi-proteins and β-arrestins. There is evidence indicating that therapeutic effect of μ-opioid receptor agonists, including analgesia, is $G\alpha_i$-protein mediated, while adverse effects, such as respiratory depression and constipation, are more related to β-arrestin recruitment (Bohn et al., 1999). In this sense, several opioid ligands were developed in order to find G-protein biased ligands with improved therapeutic effects. Recently, TRV130 (oliceridine) demonstrated *in vitro* functional selectivity for Gα-proteins, and showed to be safe for management of pain according to a phase III clinical trial (Singla et al., 2019). However, other studies pointed out that biased opioid ligands development is controversial and that further research is needed studying each drug's different signalling pathways (Gillis et al., 2020; Kliewer et al., 2020).

Another aspect to be considered for drug development is the small change in ligand structure that can result in large changes in functional selectivity profiles (Shonberg et al., 2014). For example, LSD and lisuride evoke distinct *in vitro* cellular signalling and *in vivo* responses (González-Maeso et al., 2003, González-Maeso et al., 2007), despite the structural features and shared affinity for the 5-$HT_{2A}$R. Moreover, risperidone and its active metabolite paliperidone are atypical antipsychotics, which only differ in a single hydroxyl

group. They show different pharmacological profiles that influence in their functional selectivity profile (Clarke et al., 2013). These facts highlight support the importance of deep structure-functional selectivity relation studies (Berg & Clarke, 2018).

Although many studies have shed light on ligand bias profiles of different compounds, most of the studies are limited to few signalling pathways, such as G-proteins versus β-arrestins. However, many other possibilities are feasible, and all signalling pathways should be considered when characterizing the individual ligand efficacy (**Figure 1.6**).

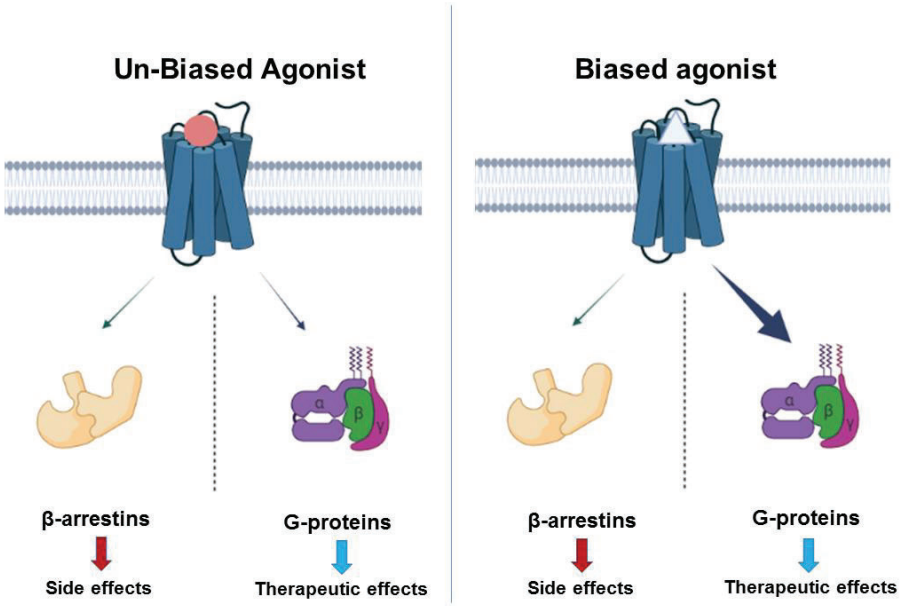



**Figure 1.6**: Schematic representation of biased agonism on GPCR. Hypothetical and simplified example of μ-opioid receptor biased agonism. G-protein signalling pathway undergoes therapeutical effect while β-arrestins cause side effects. Direct interaction of un-biased ligands would results in activation of both signalling pathways, with therapeutic and side effects. However, functional or biased agonists favour preferentially one of the signal cascades, like G-proteins, promoting therapeutic effects over side effects.

### 1.2.5 Evaluation of constitutive activity, inverse agonism and functional selectivity

GPCR implication in different diseases has increased the number of studies analysing different ligands profile to target these receptors. Because of their high relevance, several assays have been developed with a view to characterize the functional profile of different drugs.

Classical functional assays measured downstream messengers, such as $Ca^{2+}$ release, $IP_3$ accumulation or cAMP production, in order to determine the functional profile of a drug after binding a GPCR. Advantages of measuring second messengers are that unmodified receptors, in native tissues, can be

studied. These assays have been broadly used for better comparison between different publications.

Even if these techniques are widely used in assays with substantial amplification, in several assays both full and partial agonist could reach the same maximal response (Smith et al., 2018). Moreover, it is not clear the second messenger attribution to specific G-protein subtypes. Therefore, a nearest functional quantification of drug-receptor interaction was seen as necessary.

A direct evaluation of GPCR activation can be made by measuring the stimulation or inhibition of guanine nucleotide exchange on G-protein receptors using radiolabelled GTP analogues. This approach is called sulphur 35-labelled guanosine-5´-O-(γ-thio)-triphosphate ([$^{35}$S]GTPγS) binding assay. Measurement of G-protein activation is the functional consequence of receptor occupancy as earliest event, and it is not subjected to signal amplification (González-Maeso et al., 2000; Harrison & Traynor, 2003). Conventional [$^{35}$S]GTPγS binding assays are only limited to the measurement of GPCR coupling to $G\alpha_{i/o}$-proteins; probably due to their higher rates of nucleotide exchange and constitutive activity (Seifert & Wenzel-Seifert, 2002). Nevertheless, different assays have been developed in order to measure different Gα-protein responses such as [$^{35}$S]GTPγS binding immunoprecipitation assay using magnetic beads or scintillation proximity assays (SPA) (Diez-Alarcia et al., 2021b). This assay combines classical [$^{35}$S]GTPγS binding assay with immunoprecipitation. This technique can be applied for studying receptor of interest in cell cultures and in native tissues. Moreover, this assay allows the evaluation of constitutive activity using pharmacological tools, as inverse agonists, in native tissue (Diez-Alarcia et al., 2021b). Nevertheless, this [$^{35}$S]GTPγS binding assay combined with immunoprecipitation is currently limited to Gα-proteins, while β-arrestin-mediated response is not possible to be studied as yet.

In addition to previous assays, fluorescence and bioluminescence resonance energy transfer (FRET and BRET) assays, which are technologies to detect protein-protein interaction and dynamic conformational changes have been developed for directly monitoring conformational changes in GPCRs, G-proteins, and β-arrestins (Angers et al., 2000; Galés et al., 2006; Zhou et al., 2021; Wright & Bouvier, 2021). These assays are suitable for monitoring live cell events in real time, and display adaptability to high-throughput screening. Despite these advantages, the introduction of fluorescent donor and acceptor molecules needs to be considered as a factor that could influence the observed outcomes (Pottie & Stove, 2022). The use of BRET is only suitable for *in vitro* cell culture systems, whereas in live animals, the application is limited to studies in superficial locations. This assay is not suitable for the study of post-mortem tissue right now (Drinovec et al., 2012).

## 1.3 Serotonin 2A receptor (5-$HT_{2A}$R)

### 1.3.1 General aspects

5-HT regulates a wide range of physiological processes in the central nervous system (CNS), including memory, perception, cognition, emotion, mood and consciousness (Berger et al., 2009). Dysfunction of 5-HT system has been implicated in numerous psychiatric disorders (Hoyer, 2020). Therefore, pharmacological manipulation of 5-HT system has therapeutic potential. Mammalian 5-HT receptors are now classified into 14 structurally and pharmacologically distinct subtypes (Hannon & Hoyer, 2008), which are divided into seven main families (5-$HT_1$, 5-$HT_2$, 5-$HT_3$, 5-$HT_4$, 5-$HT_5$, 5-$HT_6$, 5-$HT_7$ receptors), based on operational (drug related characteristics), transductional and structural characteristics (**Figure 1.7**). At a structural level, only 5-$HT_3$ receptor is a ligand-gated ion channel (Maricq et al., 1991), whilst the rest of 5-HT receptors are members of GPCR superfamily (Kroeze & Roth, 1998).

The 5-$HT_2$ receptors are among the most widely studied 5-HT receptors. The 5-$HT_2$ receptor family is divided in three different receptors, termed as 5-$HT_{2A}$R 5-$HT_{2B}$R and 5-$HT_{2C}$R, with 46-50% sequence homology (Hoyer et al., 2002). Moreover, transmembrane domains of 5-$HT_{2A}$R and 5-$HT_{2C}$R share 80% sequence homology and very close pharmacological profiles (Boess & Martin, 1994). Thus, development of selective drugs for each receptor is a fundamental challenge due to the similar binding pockets. At present, it is not easy matter to discriminate pharmacologically 5-$HT_2$ receptor family members, due to the lack of truly selective 5-$HT_{2A}$R or 5-$HT_{2C}$R ligands.

Radioligand binding techniques were initially used for characterization of 5-HT receptors in mammalian brain homogenates. During the course of early investigations using radioligands, two classes of 5-HT binding sites were described. High affinity sites for [$^3$H]5-HT corresponded to the 5-$HT_1$ receptor

subtype, while low affinity sites were designated as 5-$HT_2$ receptor subtype. The discovery of [$^3$H]ketanserin as a reasonably selective ligand for 5-$HT_{2A}$R (Leysen et al., 1982) turned to be a huge advance, and greatly potentiated the investigation of 5-$HT_{2A}$R (Leysen et al., 1982). However, ketanserin also shows moderate affinity for 5-$HT_{2C}$R, among other receptors (Choudhary et al., 1992). During the last years, new selective 5-$HT_{2A}$R ligands have been subsequently developed for the evaluation of 5-$HT_{2A}$R, such as [$^{18}$F]altanserin, [$^3$H]MDL100907 (L´Estrade et al., 2018) and [$^{11}$C]Cimbi-36 (Ettrup et al., 2014).

Sequence analysis of 5-$HT_{2A}$R coding regions disclosed a high overall genetic conservation across species. The exception is a non-conserved change, most notably at residue 242, corresponding to a serine in humans and an alanine in rodents. It has been described that this change might influence in affinity and efficacy of a variety of 5-$HT_{2A}$R agonist (López-Giménez & González-Maeso, 2018; Kim et al., 2020; Slocum et al., 2021).

### 1.3.2 Localization and function of 5-$HT_{2A}$R in CNS

5-$HT_{2A}$R and 5-$HT_{2C}$R are both expressed in CNS. Peripherally, 5-$HT_{2A}$R is found in platelets, vascular smooth muscle cells and ocular tissue (Leysen, 2004). Rather, 5-$HT_{2B}$R is primarily found in periphery and, specifically, in human heart cardiac valves (Bonaventure et al., 2005) (**Figure 1.7**).

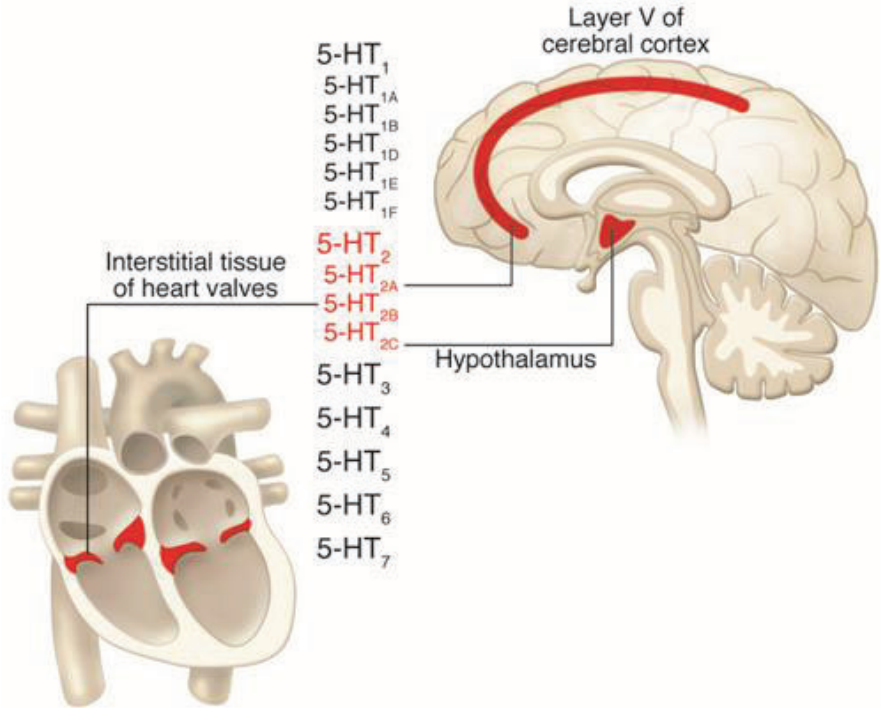



**Figure 1.7**: Schematic representation of 5-HT receptor subtype family members (5-$HT_1$ to 5-$HT_7$). 5-$HT_2$ receptor family is subdivided in three different receptor subtypes (5-$HT_{2A}$R, 5-$HT_{2B}$R and 5-$HT_{2C}$R). Each member of the 5-$HT_2$ family displays distinct brain and tissue distribution. Pyramidal neurons in layer V of cerebral cortex are enriched in 5-$HT_{2A}$R. 5-$HT_{2B}$R is present in interstitial cells of heart valves and 5-$HT_{2C}$R is localized in hypothalamus. *Illustration from Meltzer & Roth, 2013.*

Radioligand binding techniques, immunohistochemistry, electronic microscopy and *in situ* hybridization studies have made possible to explore the localization of 5-$HT_{2A}$R in CNS. Both, post-mortem autoradiography and *in vivo* neuroimaging studies, have reported high density of 5-$HT_{2A}$R in human brain (Pazos et al., 1987; Forutan et al., 2002). In human brain, 5-$HT_{2A}$R is primarily found in cortical regions (mainly prefrontal, parietal and somatosensory cortex), whereas subcortical regions and hippocampus show lower densities (Hoyer et al., 1986; Pazos et al., 1987; López-Giménez et al., 1997; Varnäs et al., 2004; Beliveau et al., 2017). More specifically, a post-mortem study

demonstrated that 5-$HT_{2A}$R are specifically present in cortical layers III and V, hypothalamus and, to a lesser extent, in hippocampus and striatal structures (Pazos et al., 1987). There is evidence that cortical 5-$HT_{2A}$R density decreases with aging (Gross-Isseroff et al., 1990; González-Maeso et al., 2008; Moses-Kolko et al., 2011; Uchida et al., 2011; Muguruza et al., 2013; Diez-Alarcia et al., 2021a).

The cellular localization of 5-$HT_{2A}$R is mainly in cortical pyramidal glutamatergic projection neurons, where they are localized at apical dendrites (Jakab & Goldman-Rackic, 1998; Miner et al., 2003; Santana et al., 2004), but also on GABAergic interneurons (Burnet et al., 1995). Generally, 5$HT_{2A}$Rs are recognized as postsynaptic receptors (relative to glutamatergic synapses) (Jakab & Goldman-Rakic, 1998). However, a proportion of 5-$HT_{2A}$R is believed to be located presynaptically in monoaminergic neuron axons (Miner et al., 2003; Bécamel et al., 2017). Additionally, 5-$HT_{2A}$R has also been identified in astrocytes and microglia (Krabbe et al., 2012; Martin & Nichols, 2016). In mammalian brain, 5-$HT_{2A}$R expression is higher in cytosolic fraction of brain homogenates than in plasma membranes (Cornea-Hebert et al., 1999; Eastwood et al., 2001). The receptor in cytoplasmic membrane is thought to be G-protein coupled and functional, as opposed to the internalized receptor.

5-$HT_{2A}$R canonically couples to $G\alpha_{q/11}$-proteins, activates downstream PLC isoform that leads to the production of $IP_3$, enhances cellular $Ca^{2+}$ release and activates PKC (see **1.3.4** for more information) (**Figure 1.8**). *In vitro* slice recordings have reported that 5-$HT_{2A}$R activation increases excitability of neurons in PFC (Araneda & Andrade, 1991). Moreover, intracortical administration of 5-$HT_{2A}$R agonist enhances neuronal excitability and neurotransmitter release (Ashby et al., 1990; Arvanov et al., 1999).

Other responses, likely mediated by 5-$HT_{2A}$R, include hyperthermia, hyperlocomotion and neuroendocrine responses, such as increased secretion of cortisol, renin and prolactin (Gudelsky et al., 1986; Barnes & Sharp, 1999;

Pytliak et al., 2011). Several studies also support that eye blink response is entirely mediated through 5-HT$_{2A}$R (Welsh et al., 1998a; Welsh et al., 1998b; Romano et al., 2000; Harvey, 2003).

The activation of 5-HT$_{2A}$R expressed in PFC has also been implicated in psychotomimetic effects of psychedelic hallucinogens like psilocybin and LSD, which are often used to model positive symptoms of schizophrenia (Vollenweider et al., 1998; González-Maeso et al., 2007; González-Maeso & Sealfon, 2009; Nichols, 2016). 5-HT$_{2A}$R is the primary target of widely used atypical antipsychotics such as clozapine, risperidone and olanzapine, which act as antagonists or even inverse agonists on this receptor (Meltzer, 1999).

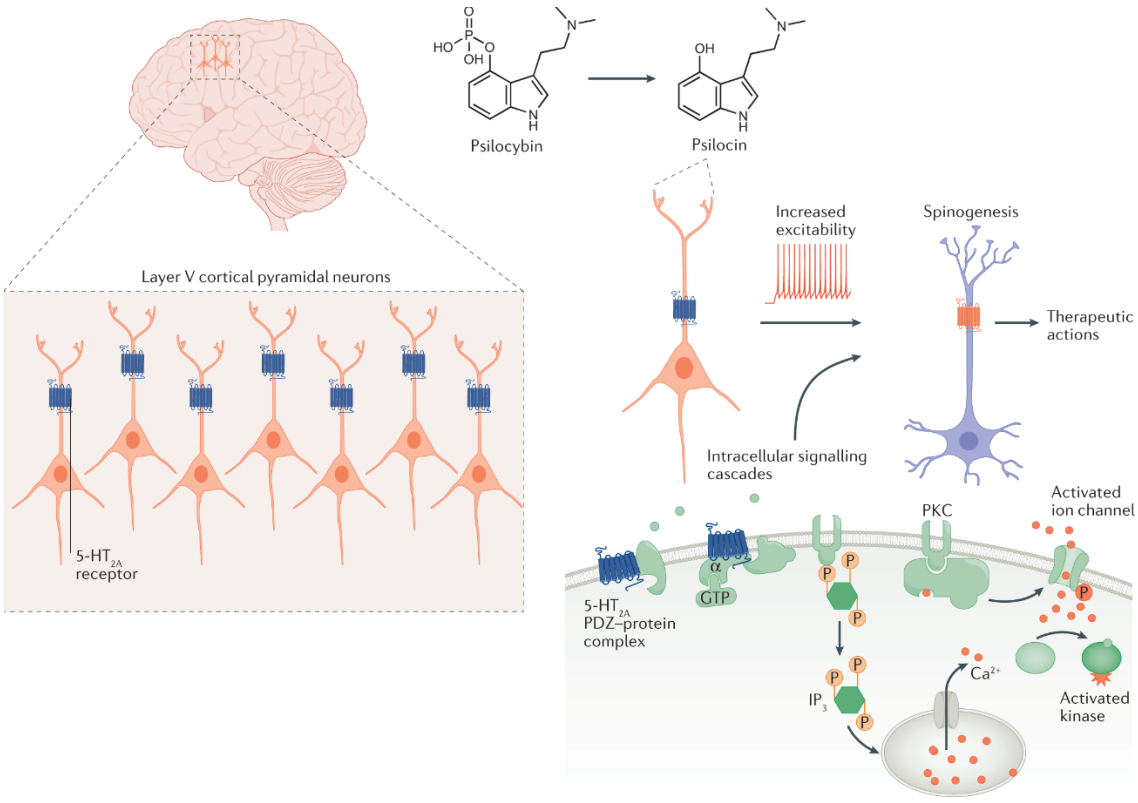



**Figure 1.8**: 5-HT$_{2A}$R are highly located in apical dendrites of pyramidal neurons. Psychedelics activate 5-HT$_{2A}$R and initiate a cascade of events leading to increased neural firing, and activating a large number of intracellular signalling cascades. *Illustration from McClure-Begley & Roth, 2022.*

### 1.3.3 5-$HT_{2A}$R Structural biology

Over the past decade, several structures of different classes of GPCRs have been determined in complex with ligands of diverse pharmacology. Pharmacophore models of ligand-receptor interactions have been developed and consequences on transmembrane conformational changes are being described (Seyedabadi et al., 2022). Homology models and crystal structures are allowing the determination of docking sites for different agonist and antagonists (Mozumder et al., 2020). Via mutagenesis of single amino acids of the target receptors and further development of functional assays, it is also possible to measure the key residues that are implicated in the coupling of the GPCR to the different Gα-proteins. In 2018 Kimura et al., found the structure of human 5-$HT_{2A}$R in complex with second generation antipsychotics risperidone and zotepine (Kimura et al., 2019). The atypical antipsychotics risperidone and zotepine effectively stabilize the inactive conformation by forming direct contacts with the residues at the bottom of the ligand-binding pocket also termed as deep binding pocket.

Among aminergic receptors, structure of the binding pocket of 5-$HT_{2A}$R is similar to that of 5-$HT_{2C}$R in complex with the inverse agonist ritanserin (Peng et al., 2018) and shows relative similarity to the structure of 5-$HT_{2B}$R in complex with the agonist ergotamine (Wacker et al., 2013). Indeed, the binding sites of risperidone and zotepine in 5-$HT_{2A}$R mostly overlap with the binding sites of ritanserin, risperidone and doxepin in 5-$HT_{2C}$R, $D_2$R and $H_1$R, respectively.

Of all conformational changes a GPCR undergoes, rearrangements of the transmembrane helices, particularly TM helices 5, 6 and 7 appear to play a critical role in transmitting a signal across the membrane (Mitra et al., 2021). Crystal structures of different GPCRs captured in both inactive and active states indicate that the global conformational changes involved in receptor activation are similar, particularly on the cytoplasmic side. However, the

specific characteristics of the 5-$HT_{2A}$R are still under evaluation. In fact, important heterogeneity in the interaction between critical amino acids and selective drugs is being described (Wacker et al., 2013; Wacker et al., 2017; Peng et al., 2018; Kim et al., 2020). Thus, very recently, it was shown that the structures of 5-$HT_{2A}$R complexed with LSD and lisuride differ from the structures of 5-$HT_{2A}$R bound to 5-HT and psilocin (Cao et al., 2022). The study revealed that ergoline moieties of LSD and lisuride are bound at the bottom of the orthosteric binding pocket, similarly to previous findings with other ligands. In contrast, the indole moiety of 5-HT and psilocin core is located closer and higher in the orthosteric binding pocket, where they engage the extended binding pocket, which is also occupied by diethyl moiety of LSD and lisuride. In summary, this study reveals a second binding mode of 5-HT and psilocin, enabling structure-based design of biased ligands (Cao et al., 2022).

Determination of the crystal structure of receptors bound to different drugs by cryoelectron microscopy also contributed to the elucidation of the mechanism underlying the functional selectivity or biased agonism of 5-$HT_{2A}$R (Che et al., 2021; Seyedabadi et al., 2022). In this sense, determination of 5-$HT_{2A}$R bound to N-(2-hydroxybenzyl)-2,5-dimethoxy-4-cyanophenylethyl-amine (25-CN-NBOH) in complex with an engineered $G\alpha_q$ heterotrimer, as well as, the structure of β-arrestin biased ligands LSD or inverse agonist methiotepin revealed the structural determinants responsible for 5-$HT_{2A}$R-$G\alpha_q$-protein interaction (Kim et al., 2020).

Overall, structure-based biased ligands discovery is revolutionizing drug development, allowing the rational design of selective and biased drug with improved clinical effects.

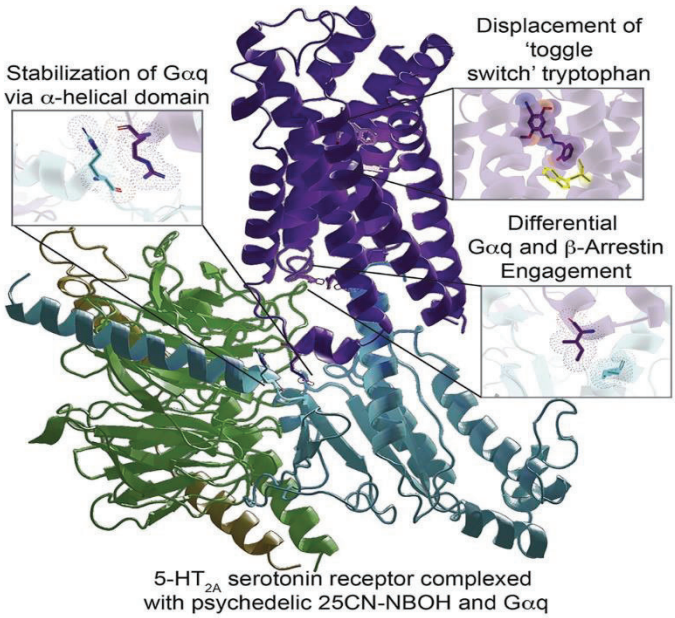



**Figure 1.9:** The overall representation cryogenic electron microscopy (Cryo-EM) structure of the 5-$HT_{2A}$R-$G\alpha_q$ bound to 25CN-NBOH. *Illustration from Kim et al., 2020*.

### 1.3.4 5-$HT_{2A}$R Signalling pathways

The 5-$HT_{2A}$R is coupled to $G\alpha_{q/11}$-proteins but may also transduce signals through alternative mechanisms, including other Gα-protein subtypes and through recruitment of β-arrestin isoforms (**Figure 1.10**). The most studied cascade and, probably, the most important signal transduction pathway regulated by this receptor is related to the activation of PLC-mediated catalysis by $G\alpha_{q/11}$-proteins, and subsequent hydrolysis of $PIP_2$ (Roth et al., 1984; Hoyer et al., 1994). This activation leads to production of inositol $IP_3$, which causes subsequent mobilization of intracellular $Ca^{2+}$, and DAG, which in turn activates PKC and calcium/calmodulin-dependent kinase II (CaMKII) (Roth et al., 1986). Then, activated PKC induces 5-$HT_{2A}$R desensitization, as well as, a number of downstream effector pathways activation such as mitogen-activated protein (MAP) kinase (MAPK) signalling (Banerjee & Vaidya, 2020).

5-$HT_{2A}$R also promotes the release of arachidonic acid (AA) through the activation of phospholipase $A_2$ ($PLA_2$) (Berg et al., 1998; Parrish & Nichols,

2006). The AA release seems to be mediated by a complex mechanism that involves RhoA signalling, MAPK and extracellular signal-regulated kinases (ERK). All of them are depending on Gα-proteins, but different from $G\alpha_{q/11}$-proteins, such as $G\alpha_{12/13}$- and $G\alpha_{i/o}$-protein subtypes (Kurrasch-Orbaugh et al., 2003a; Kurrasch-Orbaugh et al., 2003b). Several studies revealed that the 5-$HT_{2A}$R has ability to trigger the activation of $G\alpha_{i/o}$-proteins, a coupling phenomenon that underlies hallucinogenic actions caused by psychedelic drugs (González-Maeso et al., 2007).

Additionally, several phosphorylated downstream proteins have been identified by phosphoproteomic studies after $5HT_{2A}$R activation (Karaki et al., 2014). This includes ERKs, ribosomal S6 kinase (RSK-2) (Strachan et al., 2008) and arrestins (Schmid et al., 2008; Schmid & Bohn, 2010). Furthermore, 5-$HT_{2A}$R interaction with a large number of scaffolding proteins has been described, including postsynaptic density protein 95 (PSD-95) and other PSD-95/discs large/zonula-occludens 1 (PDZ)-domain-containing proteins (Xia Gray et al., 2003; Bécamel et al., 2004; Abbas et al., 2009), caveolin-1 (Bhatnagar et al., 2004; Sommer et al., 2009), and microtubule associated protein A1 (MAP1A) (Sheffler et al., 2006). Specifically, 5-$HT_{2A}$R interaction with PSD-95, caveolin-1, RSK-2 and β-arrestin2 have been described to be essential for modulation of the functional signalling.

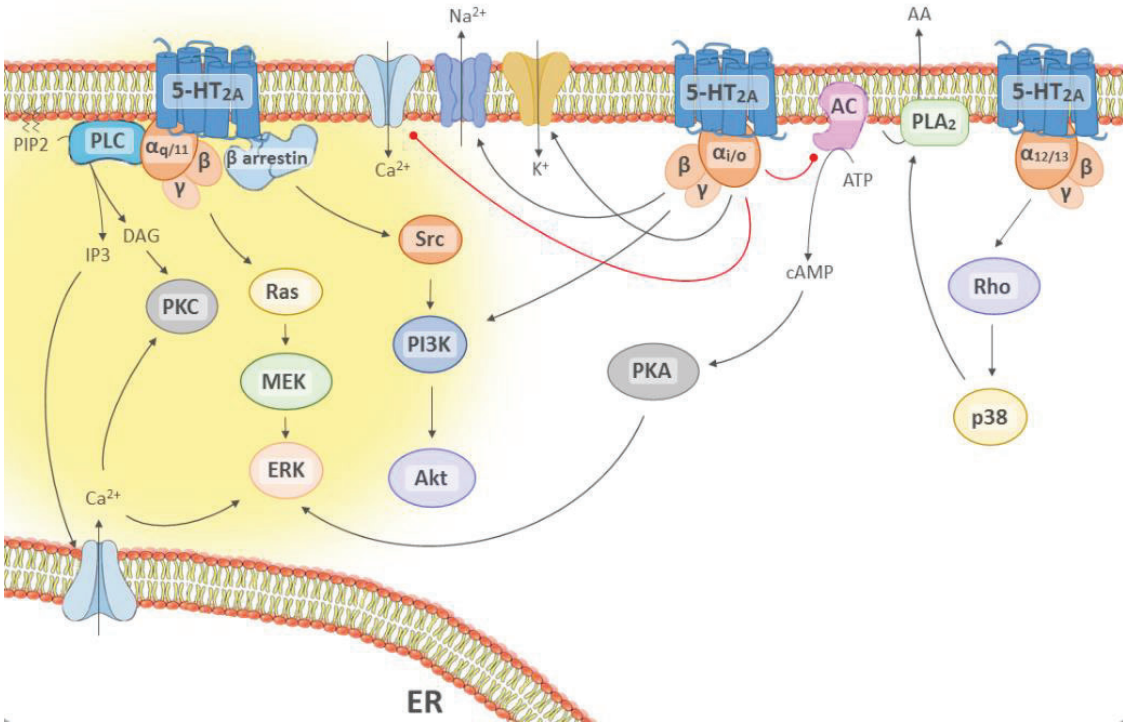



**Figure 1.10:** Illustration of the main intracellular signal transduction pathways associated with 5-HT$_{2A}$R. 5-HT$_{2A}$R signalling involves the stimulation of G$\alpha_{q/11}$-proteins, which promotes PLC-mediated catalysis of $PIP_2$ to $IP_3$ and DAG hydrolysis, thus activating PKC and elevating cytosolic $Ca^{2+}$. However, 5-HT$_{2A}$R also activates other signalling pathways. For example, it mediates AA release, presumably through the activation of $PLA_2$, mediated by a complex mechanism involving Rho and p38. All those mechanisms are depending on Gα-proteins, different from G$\alpha_{q/11}$, such as G$\alpha_{12/13}$. 5-HT is suggested to activate 5-HT$_{2A}$R and assemble proteins, such as β-arrestin2, Src and Akt. In contrast, hallucinogenic 5-HT$_{2A}$R agonists do not require this complex recruitment, and elicit differential gene regulation via G$\alpha_{i/o}$-protein subtypes. *Illustration fom Ibarra-Lecue et al., 2021.*

## 1.3.5 5-HT$_{2A}$R ligands

5-HT$_{2A}$R binding drugs belong to structurally diverse chemical classes: indolealkylamines, phenylalkylamines, arylpiperazines, alkylpiperidines, alkylpiperazines and polycyclic/tricyclic agents, among others (Westkaermper & Glennon, 2002). 5-HT$_{2A}$R drugs (either agonists, antagonists or inverse agonists) represent some of the most important drugs in neuropsychiatry,

including atypical or second-generation antipsychotics, psychedelics and antidepressant drugs (Barnes et al., 2021). Therefore, many efforts have focused on development of 5-$HT_{2A}$R selective ligands. However, many of the ligands that bind 5$HT_{2A}$R also bind 5-$HT_{2C}$R, with similar affinities, given the sequence homology found between the transmembrane portions of both receptors.

### 1.3.5.1 Agonists

5-$HT_{2A}$R agonists and partial agonists have traditionally been divided into three structural groups: ergolines (LSD, lisuride and pergolide), indolealkylamines (psilocin) (both included in indoleamines group) and phenylalkylamines (mescaline, 2,5-dimetoxy-4-iodoamphetamine ((±)DOI)) (Halberstandt & Geyer, 2011; Nichols, 2016). The physiological ligand 5-HT is a non-selective agonist that binds to all 5-HT receptors. In 5-$HT_2$ receptor family the affinity order for this molecule is 5-$HT_{2B}$R > 5-$HT_{2A}$R > 5-$HT_{2C}$R (Baxter et al., 1995).

Some of the 5-$HT_{2A}$R agonist ligands display hallucinogenic effects in humans (LSD, psilocybin, psilocin and mescaline). In this sense, classic hallucinogens or serotonergic psychedelics are described as substances capable of altering thoughts, perception, and mood, via activation of 5-$HT_{2A}$R (Glennon et al., 1984; Vollenweider et al.,1998; González-Maeso et al., 2007; Halberstadt, 2015; Madsen et al., 2019). Despite this effects has been reported to be mediated by 5-$HT_{2A}$R, classic hallucinogens are not selective and bind other receptors.

In this sense, phenylalkylamine hallucinogens are selective for 5-$HT_2$ receptors, including 5$HT_{2A}$R, 5$HT_{2B}$R and 5-$HT_{2C}$R. Among the different molecules, (±)DOI has been described as a potent but non-specific ligand for 5-$HT_{2A}$R and 5-$HT_{2C}$R. (±)DOI has often been the agonist of choice for *ex vivo/in vivo* studies probing 5-$HT_{2A}$R-mediated functions (Nelson et al., 1999;

Pigott et al., 2012; Canal et al., 2013). [$^{35}$S]GTPγS experiments in human post-mortem brain and 5-HT$_{2A}$R knock-out animals confirmed that (±)DOI behaves as 5-HT$_{2A}$R and 5-HT$_{2C}$R partial agonist (Diez-Alarcia et al., 2019; Garcia-Bea et al., 2019; Muneta-Arrate et al., 2020).

During the last years, a new class of ligands have been developed with higher affinity on 5HT$_{2A}$R, based on N-benzylphenethylamine (NBOMe) scaffold such as n-(2-methoxybenzyl)-2,5-dimethoxy-4-bromophenyletylamine (25B-NBOMe, Cimbi-36) and 25-CN-NBOH (Jensen et al., 2020). These molecules are suitable tools for PET imaging and pharmacological studies. 25-CN-NBOH and Cimbi-36 show partial agonist activity, exhibiting higher affinity for 5-HT$_{2A}$R vs 5-HT$_{2C}$R and 5-HT$_{2B}$R (Hansen et al., 2014; Jensen et al., 2017).

On the other hand, indolealkylamines like psilocin (the active metabolite of psilocybine) and ergolines, like LSD, are relatively non-selective 5-HT receptors ligands, displaying moderate to high affinity for 5-HT$_1$ and 5-HT$_2$ receptors (Roth, 2007; Nichols, 2016; Wacker et al., 2017). Moreover, LSD binds with high affinity to other 5-HT receptors, but also to dopamine receptors (Halbertadt & Geyer, 2011; Borroto-Escuela et al., 2014).

Head-twitch response (a rapid side-to-side movement of head) represent the most widely used rodent response to evaluate hallucinogenic effects induced by psychedelics through 5-HT$_{2A}$R. This is considered a critical test for *in vivo* evaluation of functional response to psychedelics. Several studies using selective antagonist and 5HT$_{2A}$R knock-out animals have demonstrated that head-twitch response is very selective of 5-HT$_{2A}$R, and is limited to psychedelics (González-Maeso et al., 2007; Canal & Morgan, 2012). However, chemically closely related 5-HT$_{2A}$R agonist like lisuride, ergotamine and pergolide (González-Maeso et al., 2003; González-Maeso et al., 2007) does not induce this behaviour. Lisuride and pergolide are termed non-hallucinogenic 5-HT$_{2A}$R agonists. Those compounds, as other ergolines, present a very complex polypharmacological profile, showing affinity for

several aminergic receptors, including serotonergic and dopaminergic receptors (Halberstadt & Geyer, 2011). Moreover, lisuride and pergolide are considered antiparkinsonian drugs, due to their high affinity for dopamine receptors and 5-$HT_{1A}$R (Langtry & Clissold, 1990; Marona-Lewicka et al., 2002).

### 1.3.5.2 Antagonists

One of the largest and selective classes of 5-$HT_{2A}$R antagonist are the N-alkylpiperidones, being ketanserin the most widely used for years. Ketanserin is selective for 5-$HT_{2A}$R vs 5-$HT_{2C}$R (15-80 fold) and 5-$HT_{2B}$R (500-1000 fold) (Jerman et al., 2001; Knight et al., 2004; Diez-Alarcia et al 2019). Chemically, ritanserin is close to ketanserin, is highly potent, relatively selective and long acting 5-$HT_{2A}$R antagonist, but is also described as inverse agonist (Bonhaus et al., 1995). Another 5-$HT_{2A}$R antagonist, chemically related to ketanserin, is the benzoylperidine altanserin, that has been described as potent and selective 5-$HT_{2A}$R antagonist with a 20-fold greater affinity for human 5-$HT_{2A}$R versus human 5-$HT_{2C}$R (Tan et al., 1999). However, inverse agonist properties on 5-$HT_{2A}$R have been previously reported for altanserin (Aloyo et al., 2009; Diez-Alarcia et al., 2019).

Other 5-$HT_{2A}$R-selective (or preferring) ligands have been developed such as MDL100907, also known as volinanserin, and MDL-11,939. Volinanserin is a potent 5-$HT_{2A}$R antagonist and shows 300-fold selectivity for 5-$HT_{2A}$R receptor versus 5-$HT_{2C}$R and other GPCRs (Sorensen et al., 1993; López-Giménez et al., 1998). Volinanserin and ritanserin were evaluated as antipsychotics for schizophrenia; however, they turn not to be successful (Jones et al., 2020). Nowadays, volinanserin is used as reference 5-$HT_{2A}$R antagonist due to its high selectivity.

Recently, new drugs as nelotanserin and eplivanserin have been developed for insomnia treatment. These compounds show high affinity for 5-$HT_{2A}$R with 20-fold selectivity versus 5-$HT_{2C}$R (Rinaldi-Carmona et al., 1992; Al-Shamma et al., 2010).

Pimavanserin, also known as ACP-103, is described as a highly selective drug for 5-$HT_{2A}$R, lacking affinity for other receptors except 5-$HT_{2C}$R (30-fold lower selectivity), and no significant activity on any other GPCR (Vanover et al., 2006; Abbas & Roth, 2008). The US Food and Drug Administration (FDA) has approved this drug for hallucinations and delusions treatment associated with Parkinson´s disease psychosis (Cummings et al., 2014).

Some other 5-$HT_{2A}$R antagonist, although selective for 5-$HT_{2A/2C}$ receptors bind with modest to high affinity to dopaminergic, histaminergic, and/or adrenergic receptors. Thus, atypical antipsychotics (e.g. risperidone, clozapine and olanzapine) and tricyclic antidepressants (e.g. amitriptyline, clomipramine, and imipramine) also bind to $5HT_{2A}$R as antagonist (Roth et al., 2004; Meltzer & Massey, 2011; Meltzer, 2012).

### 1.3.6 5-$HT_{2A}$R functional selectivity

5-HT receptors, specifically 5-$HT_{2A}$R, were among the first GPCR for which occurrence of functional selectivity was suggested (Berg et al., 1998). Several studies support that drugs with high affinity to 5-$HT_{2A}$R (both agonist and antagonist) stabilize distinct receptor conformations. This fact lead to biased interactions or functional selectivity with various downstream effectors, which include the canonical $G\alpha_{q/11}$-protein and non-canonical signalling (López-Giménez & González-Maeso, 2018). In this sense, biased agonism has been proposed to explain the fact that hallucinogenic and non-hallucinogenic drugs activate the same population of cortical 5-$HT_{2A}$R, but they differ in Gα-protein regulation, transcriptome fingerprints, electrophysiological responses, as well

as, behavioural states (González-Maeso et al., 2003; González-Maeso et al., 2007; Karaki et al., 2014; Banerjee & Vaidya, 2020).

One of the first evidence of functional selectivity arose from the finding of Berg and co-workers, regarding ability of this receptor to signal not only via $G\alpha_q$-dependent PLC activation, but also via $PLA_2$ (Berg et al., 1998). Specifically, PLC-dependent IP increase and AA release, via $PLA_2$ activation, were measured to demonstrate the difference of efficacies depending on which signal transduction pathway was activated. According to those results, 5-HT preferentially activated PLC-IP pathway, whereas LSD favoured $PLA_2$-AA pathway (Berg et al., 1998; Martí-Solano et al., 2015).

Hallucinogenic and non-hallucinogenic 5-$HT_{2A}$R agonists also differentially influence gene expression patterns (González-Maeso et al., 2003; González-Maeso et al., 2007). This approach were tested in cells and mouse somatosensory cortex, showing different transcriptome fingerprint between hallucinogenic and non-hallucinogenic 5-$HT_{2A}$R agonists. Thus, both hallucinogenic and non-hallucinogenic drugs induced c-fos expression. However, transcripts egr-1 and erg-2 were activated by hallucinogens-like (±)DOI and LSD, but expression of these two genes was unaffected by non-hallucinogenic agonists (lisuride and ergotamine) (**Figure 1.11**). These results also indicated that all 5-$HT_{2A}$R agonists activated 5-$HT_{2A}$R coupled to PLC, whereas hallucinogenic-dependent response involved PTX-sensitive heterotrimeric $G\alpha_{i/o}$ proteins. Notably this hypothesis was validated by quantitative phosphoproteomic approach (Karaki et al., 2014). This hypothesis was strongly supported by comparison between functional selectivity profile of the hallucinogenic drug (±)DOI with the non-hallucinogenic drug pergolide in post-mortem human brain cortex. Both 5-$HT_{2A}$R agonists induced $G\alpha_{q/11}$-protein activation, while $G\alpha_{i1}$-protein stimulation was only limited to the hallucinogenic drug (±)DOI (Muneta-Arrate et al., 2020). In conclusion, hallucinogenic agonists, such as LSD and psilocybin, promote activation of

canonical $G\alpha_{q/11}$-protein cascade as well as a $G\alpha_{i/o}$ protein-mediated signalling pathway. In contrast, chemically analogous 5-$HT_{2A}$R agonists lacking of hallucinogenic properties, like lisuride, ergotamine and pergolide, only stimulate $G\alpha_{q/11}$-protein-dependent pathway.

Other study revealed a distinct signalling signature between hallucinogenic and non-hallucinogenic 5-$HT_{2A}$R agonists. The authors noted higher levels of phospho-PLC, pERK, pCaMII, pCREB as well as higher levels of IP and DAG production after receptor stimulation with (±)DOI than those observed with lisuride (Banerjee & Vaidya, 2020).

Additionally, 5-$HT_{2A}$R activates other signal transduction cascades, such as β-arrestin, besides G-protein mediated PLC-β pathway, in a ligand-dependent manner. 5-HT and (±)DOI can differentially activate 5-$HT_{2A}$R in cellular models and *in vivo* head-twitch response. By using mice lacking β-arrestin-2, the absence of head-twitch response as demonstrated in presence of 5-HT. Akt phosphorylation also seems to be present after activation of β-arrestin-2, whereas is absent in presence of (±)DOI. However, the hallucinogenic drug (±)DOI seems to mediate head-twitch response independent of β-arrestin-2. In conclusion, these structurally distinct agonists elicit different signal transduction and trafficking patterns upon 5-$HT_{2A}$R activation (Schmid et al., 2008; Schmid & Bohn, 2010).

Recently, it was suggested that head-twitch response to the hallucinogenic drug LSD was β-arrestin-2-dependent and β-arrestin-1-independent (Rodriguiz et al., 2021). However, non-hallucinogenic drugs that bind to 5-$HT_{2A}$R stimulated β-arrestin-2 recruitment, in contrast to previous findings (Cao et al., 2022). Therefore, implication of β-arrestin-2 in the hallucinogenic response of 5-$HT_{2A}$R agonists needs further research.

On the other hand, the atypical antipsychotic clozapine inhibits 5-$HT_{2A}$R signalling through a G-protein-dependent mechanism. It induces receptor

internalization and Akt phosphorylation, regardless of receptor interaction with β-arrestin-2. Thus, 5-HT and clozapine use distinct molecular mechanisms to achieve the same 5-$HT_{2A}$R mediated downstream events: Akt phosphorylation and receptor internalization. This way, Akt phosphorylation is required for clozapine-mediated effects suppression, when this is studied on schizophrenic-like behaviours induced by MK-801 and PCP administration in mice (Schmid et al., 2014)

The complexity of biased signalling to elicit selective functional responses has provided an alternative avenue to develop novel therapeutics with increased clinical effect and less side effects. However, the use of biased agonism and other pharmacological properties, such as inverse agonism or antagonism, remains questionable for development of new therapies for schizophrenia and depression.

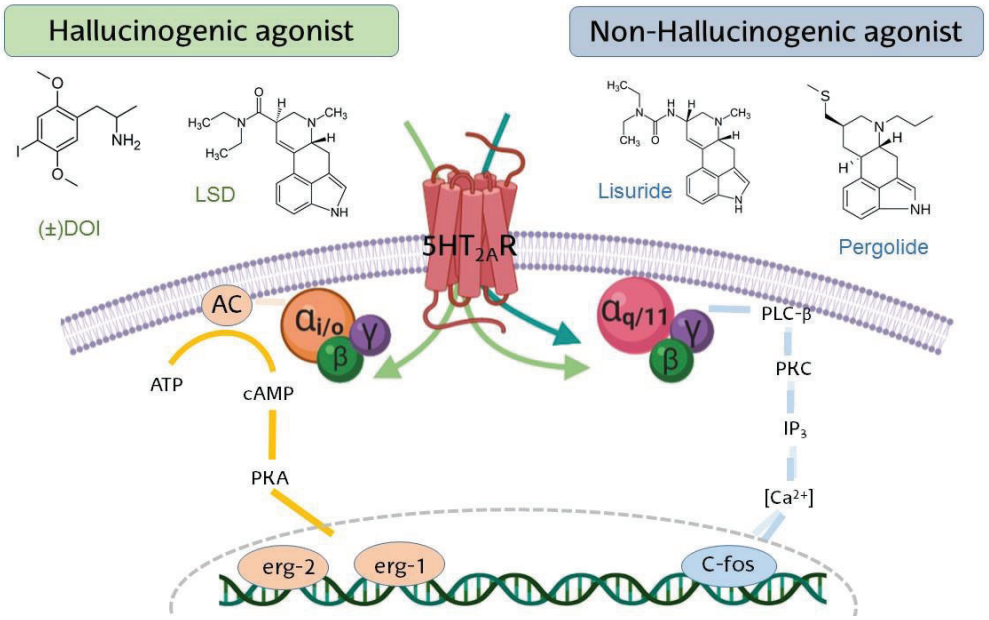



**Figure 1.11**: Schematic intracellular signalling pathways of 5-$HT_{2A}$R coupling to their downstream effectors.

## 1.4 5-$HT_{2A}$R and schizophrenia

Several reports have focused on the relation between 5-$HT_{2A}$R and schizophrenia. Despite determination of the exact role of 5-HT plays has proven elusive (Halberstadt & Geyer, 2013), several studies have focused on 5-$HT_{2A}$R because atypical antipsychotics block this receptor with high affinity (Meltzer et al., 1989; Miyamoto et al., 2005). Moreover, as indicated before, 5-$HT_{2A}$R mediates psychotic-like states exerted by some drugs, such as psilocybin, LSD or (±)DOI, both in humans and rodents (Vollenwider et al., 1998; González-Maeso et al., 2007).

### 1.4.1 Genetic studies

It has been proposed some genetic associations of HTR2A gene variants and schizophrenia. In this way, mutations in this gene and/or its promoter are associated with schizophrenia, although none of them seems to replicate consistently over distinct populations. Indeed GWAS carried out in schizophrenia did not find statistical significance for any variation of this gene (Farrell et al., 2015).

Alterations of HTR2A expression in schizophrenia have been related with three different Single Nucleotide Polymorphisms (SNPs): A-1438G in promoter region (Ohara et al., 1998), His452Tyr in coding region (Ozaki et al., 1996), and T102C in exon 1 (Arranz et al., 1996). Even if several studies support the involvement of these SNPs in schizophrenia, other studies present opposite information.

Some studies have suggested A-1438G polymorphism is associated with susceptibility to schizophrenia (Parsons et al., 2004; Peñas-Lledo et al., 2007; Sáiz et al., 2007; Smith et al., 2013). A-1438G has also been shown to interfere in response to antipsychotic treatment (Yan et al., 2021).

On the other hand, T102C (rs6313) has also been associated with schizophrenia in different populations, as well as to early response to risperidone and olanzapine in schizophrenia patients (Petronis et al., 2000; Maffioletti et al., 2020).

Finally, His452Tyr (rs6314) was described as associated with schizophrenia, but even more with antipsychotic response. The variable clinical response to clozapine and olanzapine has been related to this polymorphism (Birkett et al., 2000; Olajossy-Hilkesberger et al., 2011). Moreover, HTR2A rs6314 affects 5-$HT_{2A}$R expression and functionality, contributing to modulation of endophenotypes of schizophrenia like cognitive behaviours and related prefrontal activity (Blasi et al., 2013).

The inconsistency of data regarding the functional consequences of these SNPs on 5-$HT_{2A}$R function, expression or antipsychotic response warrants further investigations in preclinical and clinical studies.

### 1.4.2 Evaluation of 5-$HT_{2A}$R density, expression and functionality in schizophrenia subjects.

The mRNA expression of 5-$HT_{2A}$R has been evaluated in post-mortem brain of schizophrenia subjects yielding different outcomes. In PFC of schizophrenia subjects, both a decrease and no changes in 5-$HT_{2A}$R mRNA expression have been reported (Burnet et al., 1996; Hernandez & Sokolov, 1997; Hernandez & Sokolov, 2000; Lopez-Figueroa et al., 2004).

More recently, 5-$HT_{2A}$R mRNA expression was evaluated in post-mortem PFC tissue of antipsychotic-free and antipsychotic-treated schizophrenia subjects. According to the results, 5-$HT_{2A}$R mRNA expression in antipsychotic-free schizophrenia subjects was similar to controls. In contrast, in antipsychotic treated schizophrenia subjects, lower mRNA expression was observed (Garcia-Bea et al., 2019). These results were further supported by another

study in post-mortem brain of treated schizophrenic subjects, which also showed a decrease in 5-$HT_{2A}$R mRNA expression (Zhao et al., 2022).

Several reports have shown changes in 5-$HT_{2A}$R expression and density in post-mortem human tissue from subjects with schizophrenia, showing conflicting results. 5-$HT_{2A}$R density is typically assessed *in vivo* using PET, and *in vitro* using post-mortem tissue homogenates and sections. Thus, while some post-mortem studies supported increased 5-$HT_{2A}$R density, other observed no changes, and even decreases.

These apparent discrepancies may depend on several confounding issues. Recent reviews discuss a possible role of demographic and clinical measures in the opposite results obtained among post-mortem studies such as age, treatment with antipsychotics or other psychotropic drugs, and suicide as cause of death (Dean, 2003; González-Maeso & Sealfon, 2009). Moreover, it has also been suggested that methodological factors, such as the radioligand used or the sample preparation, might result in different outcomes, when using post-mortem brain from schizophrenia subjects (Dean et al., 2008; Diez-Alarcia et al., 2021a).

Early post-mortem studies were made using the non-selective 5-$HT_{2A}$R partial agonist LSD, and reported a significant decrease in different cortical regions of schizophrenia subjects (Perotuka & Snyder, 1979). However, other studies reported opposite results using [$^{3}$H]LSD (Joyce et al., 1993; Gurevich & Joyce, 1997). More recently, a newer study revealed an increased binding of [$^{3}$H]LSD to 5-$HT_{2A}$R (Diez-Alarcia et al., 2021a).

On the other hand, in presence of [$^{18}$F]setoperone and [$^{18}$F]-methyl spiperone increases, decreases or even no changes were found (Trichard et al., 1998; Ngan et al., 2000; Verhoeff et al., 2000; Okubo et al., 2000). It should be noted that these radiotracers also display important affinity for dopamine receptors, which have been also implicated in schizophrenia.

Conversely, *in vivo* PET studies as well as *in vitro* binding studies in post-mortem brain using [$^{18}$F]altanserin, a more selective 5-HT$_{2A}$R radiotracer, lead to the opposite conclusion. Those studies suggest a decreased 5-HT$_{2A}$R density in the brain cortex of antipsychotic-free schizophrenia subjects (Erritzoe et al., 2008; Rasmussen et al., 2010; Rasmussen et al., 2016; Diez-Alarcia et al., 2021a).

In addition, several studies have assed post-mortem cortical 5-HT$_{2A}$R density by using [$^{3}$H]ketanserin. Studies using [$^{3}$H]ketanserin have shown a decrease or no changes in cortical 5-HT$_{2A}$R density of subjects with schizophrenia under antipsychotic treatment (Dean et al., 1998; Pralong et al., 2000; Dean et al., 2008). 5-HT$_{2A}$R down-regulation has been described after chronic treatment with the atypical antipsychotic clozapine (Garcia-Bea et al., 2019). In contrast, most revealing studies using [$^{3}$H]ketanserin and accounting for antipsychotic presence, have shown increased 5-HT$_{2A}$R density, but only in those subjects which tested negative for antipsychotics in blood at the time of death (González-Maeso et al., 2008, Muguruza et al., 2013).

Finally, [$^{11}$C]MDL100907 has been widely used as selective 5-HT$_{2A}$R antagonist radiotracer for PET studies (L´Estrade et al., 2018). Surprisingly, *in vitro* study in post-mortem human brain with [$^{3}$H]MDL100907 showed no changes in 5-HT$_{2A}$R density (Diez-Alarcia et al., 2021a).

From a pharmacological point of view, these apparent discrepant conclusions can be unified. As previously mentioned, 5-HT$_{2A}$R display active (coupled to G-protein) and inactive (uncoupled to G-proteins) molecular conformations (Battaglia et al., 1984; López-Giménez et al., 2001) (**Figure 1.12**). Selective antagonist radiotracers bind with same affinity to both receptor conformations. In contrast, agonist and inverse agonists display a preference for specific subpopulations of each receptor conformation (active and inactive conformational states, respectively) (Kenakin, 2011). In this sense, for a better interpretation of the results, a re-evaluation of pharmacological properties of

the different radiotracers should be considered attending to their pharmacological profiles.

Thus, even if altanserin and volinanserin were initially described as antagonists, altanserin has recently been described as inverse agonist in human post-mortem brain (Diez-Alarcia et al., 2019). In contrast, ketanserin, usually assumed to be antagonist, was described as partial agonist (Muguruza et al., 2013). Therefore, altanserin could bind preferentially to the low affinity conformational state of 5-$HT_{2A}$R, while LSD as well as ketanserin would preferentially recognise the high-affinity state.

An *in vitro* post-mortem study in schizophrenia subjects shed light on 5-$HT_{2A}$R density in schizophrenia (Diez-Alarcia et al., 2021a). This study observed in schizophrenia subjects a higher proportion of 5-$HT_{2A}$R-active functional conformation, which is rather identified by agonist radiotracers. A consequent reduction of the inactive conformation, which is preferentially identified by inverse agonist radiotracers, was also confirmed. As expected, antagonist radiotracers did not distinguish between different conformational states of the receptor, with a subsequent lack of change in the receptor density.

Cortical 5-$HT_{2A}$R from schizophrenia subjects show overactive trafficking, specifically towards $G\alpha_{i1}$-proteins, whereas the canonical $G\alpha_{q/11}$ signalling pathway of this receptor remains unaltered (Garcia-Bea et al., 2019; Odagaki et al., 2021). Based in previously mentioned radioligand binding studies and these functional findings, increased 5-$HT_{2A}$R sensitivity should not be a consequence of higher receptor synthesis or expression, as previously assumed. In fact, 5-$HT_{2A}$R alteration in schizophrenia subjects seems to be more related with an altered conformational exchange between G-protein-coupled (active) and G-protein-uncoupled receptor states (Muguruza et al., 2013; Diez-Alarcia et al., 2021a).

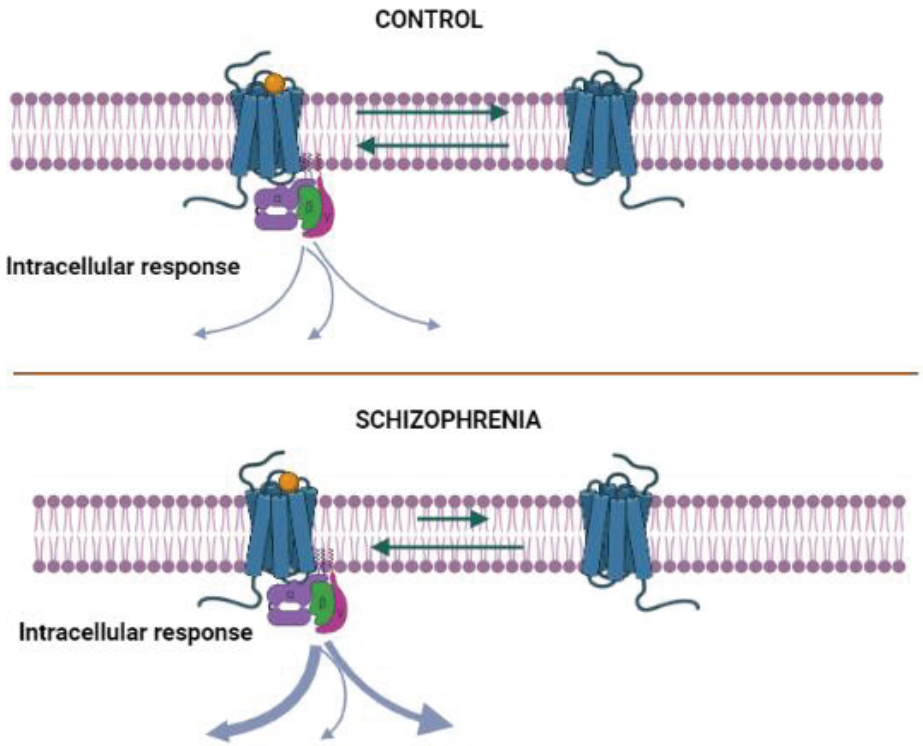



**Figure 1.12:** Representation of the imbalance between 5-$HT_{2A}$R conformations and the displaced equilibrium towards the state preferentially labelled by agonist in prefrontal cortex of schizophrenia subjects. *Illustration adapted from Diez-Alarcia et al., 2021a.*

## 1.5 Antipsychotics

Antipsychotics are used as first line medication to treat schizophrenia, and are best classified into two categories: First-generation antipsychotics or typical antipsychotics (e.g., haloperidol, chlorpromazine) and second-generation antipsychotics or atypical antipsychotics (e.g., clozapine, risperidone).

Existing medication to treat schizophrenia is effective for treating positive symptoms but have little impact on negative or cognitive symptoms (Conn et al., 2008; Leucht et al., 2009). This fact usually contributes to poor functional outcome. Consequently, there is an urgent need to identify new molecular targets and to develop mechanistically novel compounds for more effective and better-tolerated antipsychotic agents that could improve the therapeutic effects and the safety profile.

### 1.5.1 First-generation of antipsychotics (Typical antipsychotics)

The mechanism of action of typical antipsychotics is the antagonism of dopamine $D_2R$, $D_3R$ and/or $D_4Rs$. This finding led to the hypothesis that there is a dysregulation of dopaminergic system that corresponds to a hyperactivity of mesolimbic pathway, and a hypofunction of mesocortical pathway. Thus, blockade of $D_2R$ is associated with a reduction of dopamine and psychotic symptoms (Seeman, 1992; Marder et al., 1993). The blockade of $D_2R$ is also related to extrapyramidal side effects and hyperprolactinemia (Miyamoto et al., 2008).

Haloperidol is the prototypical typical antipsychotic (**Figure 1.13**). Typical antipsychotics have the ability to reduce positive symptoms and risk for relapse, improving clinical outcomes for many patients with schizophrenia. However, near 30% of patients have little or no response to typical

antipsychotics and also null benefit for negative symptoms or cognitive impairment (Conley & Kelly, 2001; Legge et al., 2020).

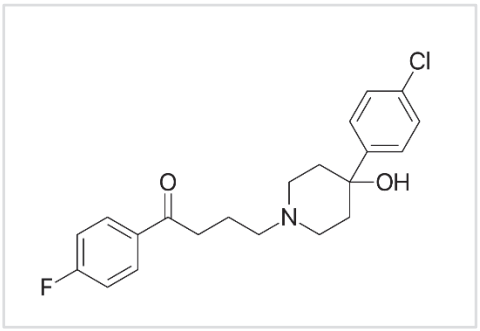



**Figure 1.13**: Chemical structure of haloperidol.

## 1.5.2 Second-generation of antipsychotics (Atypical antipsychotics)

Second-generation or atypical antipsychotics were developed looking for reduce extrapyramidal side effects at therapeutically effective doses (**Figure 1.14**). Atypical antipsychotics display higher affinity for 5-$HT_{2A}$R comparing to $D_2$R family, which explains lower extrapyramidal effects (Meltzer et al., 1989; Farde et al., 1992). Hence, 5-$HT_{2A}$R-$D_2$R activity ratio has been considered to be more relevant to predict a reduce side effect liability than improved efficacy (Ebdrup et al., 2011). Unfortunately, second-generation antipsychotics have an increased risk of weight gain, and disturbances in glucose and lipid metabolism (Muench & Hamer, 2010; Weston-Green et al., 2013; Grajales et al., 2019).

The discovery of clozapine contributed to the introduction of new drugs with more beneficial pharmacological profile than first-generation antipsychotics (Meltzer et al., 1989). The complex pharmacological profile of clozapine has made the task of determining its mechanism of action extremely difficult. Clozapine has reasonable affinity for a large number of receptors, including

several histaminergic, serotonergic, adrenergic, dopaminergic and cholinergic subtype (Coward, 1992; Nucifora et al., 2017). In addition to activity on dopamine and 5-$HT_{2A}$R, clozapine is also 5-$HT_{1A}$R partial agonist, which is thought to be beneficial in terms of reducing cognitive and negative symptoms. Muscarinic receptors are also affected by clozapine, by blocking $M_1$, $M_2$, $M_3$ and $M_5$ receptors ($M_1$R, $M_2$R, $M_3$R, $M_5$R), while stimulated $M_4$ receptor ($M_4$R). Moreover, clozapine antagonizes histamine receptors, which is related to sedation effects. Clozapine also blocks adrenergic receptors, which causes hypotension and tachycardia (Coward et al., 1992; Nucifora et al., 2017).

Clozapine is also associated with an elevated risk of potentially lethal hematotoxicity (agranulocytosis and neutropenia), an adverse effect that restricts its clinical use (Alphs et al., 1991). Consequently, new second-generation antipsychotics were introduced, such as risperidone, olanzapine, quetiapine, among others, with an effort to reduce side effects related to blood dyscrasias.

Olanzapine is a chemical analogue of clozapine with similar pharmacological properties. However, it is not associated with a risk of agranulocytosis. As expected, olanzapine presents higher affinity to 5-$HT_{2A}$R than to DA receptors. It also blocks histamine, muscarinic and adrenergic receptors but is weaker comparing with clozapine. Weight gain and sedation are the most frequent side effect of olanzapine (Fulton & Goa, 1997; Leucht et al., 2013).

Quetiapine acts as $D_1$R, $D_2$R and 5-$HT_{2A}$R antagonist as well as 5-$HT_{1A}$R partial agonist. Side effects induced by quetiapine are associated to $\alpha_1$-adrenergic and histaminergic antagonism (Miodownik & Lerner, 2006).

Risperidone is another atypical antipsychotic drug. Therapeutic effect of risperidone results from both $D_2$R and 5-$HT_{2A}$R antagonism, showing stronger affinity for 5-$HT_{2A}$R than for $D_2$R (Cohen, 1994). Moreover, this drug also causes $\alpha_1$-adrenergic and histamine receptor blockade. Risperidone is not only

efficient in treating positive symptoms, but also negative and cognitive disturbances. This fact makes it one of the most commonly prescribed antipsychotics (Möller, 2005; Chopko & Lindsley, 2018).

Paliperidone is an active metabolite of risperidone, which acts at the same receptors range.

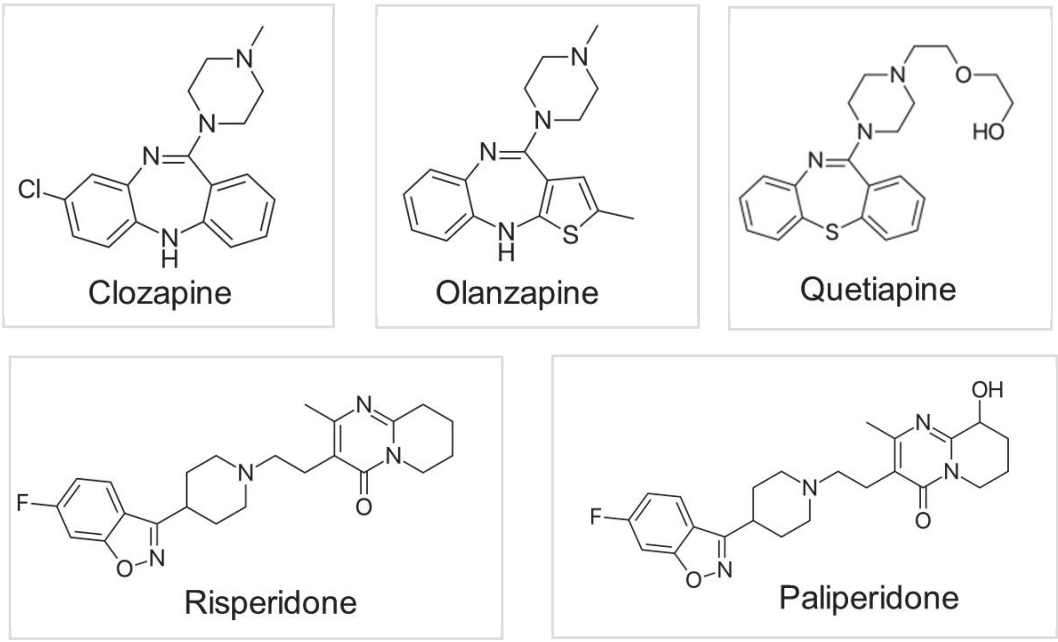



**Figure 1.14:** Chemical structure of clozapine, olanzapine, quetiapine, risperidone and paliperidone.

In the last years, new second-generation antipsychotics have been developed like asenapine and lurasidone (Miyamoto et al., 2012). However, the improved efficacy with respect to first-generation antipsychotics has yet to be determined. Right now, clozapine is the only drug approved for resistant schizophrenia treatment (Conley & Kelly, 2001).

## 1.5.3 Third-generation of antipsychotics

Another generation of antipsychotics, such as aripiprazole, have also been developed. This group of drugs is known as third-generation of antipsychotics (**Figure 1.15**). Unlike other neuroleptics, third-generation antipsychotics are not $D_2R$ antagonist but $D_2R$ partial agonist (Davies et al., 2004). In high concentrations of DA, those antipsychotics compete with DA, and results in partial antagonism, leading to clinical benefits. Contrary, when DA levels are low, aripiprazole can bind to $D_2R$ and act as partial agonist. Moreover, aripiprazole also shows partial agonist properties to 5-$HT_{1A}R$. Contrary to second-generation antipsychotics, aripiprazole shows higher affinity for $D_2R$ than for 5-$HT_{2A}R$ (Chen et al., 2022). Third-generation antipsychotics are effective in alleviating psychotic symptoms without inducing extrapyramidal side effects and hyperprolactinemia, as well as lower weight gain and metabolic liabilities (Lieberman, 2004).

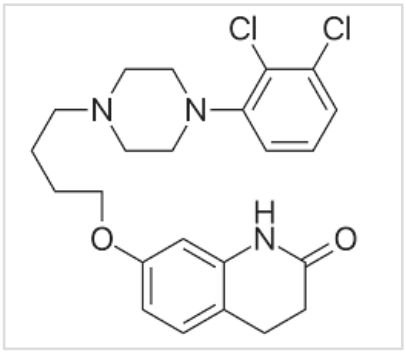



**Figure 1.15**: Chemical structure of aripiprazole.

## 1.5.4 New generation of antipsychotics

Different efforts to find new therapies for schizophrenia based on the use of 5-$HT_{2A}R$ antagonists, like ritanserin and volinanserin, have failed to be therapeutically useful. Due to the lack of efficacy of monotherapy with selective 5-$HT_{2A}R$ antagonist, different researches indicate that 5-$HT_{2A}R$ antagonism alone is not enough to explain the efficacy of atypical antipsychotics (Miyamoto

et al., 2012). Thus, $D_2R$ blockade seems to be necessary. However, a potent and selective 5-$HT_{2A}R$ drug, pimavanserin, was developed as a new alternative for the treatment of psychosis (**Figure 1.16**) (Meltzer & Roth, 2013). Pimavanserin is the first approved antipsychotic that lacks of dopaminergic affinity (Hacksell et al., 2014). Until now, pimavanserin has been tested as adjunctive therapy for schizophrenia in combination with haloperidol and risperidone (Meltzer et al., 2012), and has gained FDA approval to reduce delusions and hallucinations in Parkinson's disease (Cummings et al., 2014). Moreover, pimavanserin has shown ability to reduce negative symptoms in schizophrenia patients (Bugarski-Kirola et al., 2022). Pimavanserin has been described as 5-$HT_{2A}R$ inverse agonist. However, evidence of this aspect needs to be demonstrated (Vanover et al., 2006; Nutt et al., 2017).

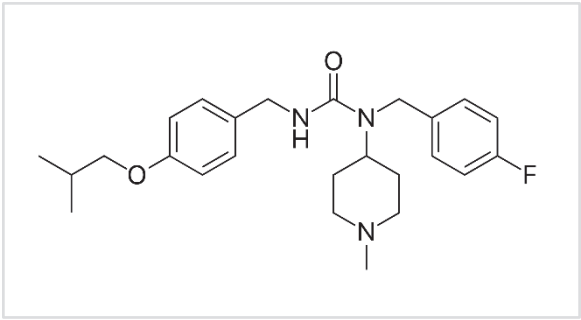



**Figure 1.16:** Chemical structure of pimavanserin.

It has been recently recognized that many second-generation antipsychotics are 5-$HT_{2A}R$ inverse agonists rather than neutral antagonists (Weiner et al., 2001). In contrast to antagonists, inverse agonists possess negative intrinsic efficacy, and can attenuate basal constitutive signalling activity.

Overall, a better understanding of the mechanism of action of antipsychotics could lead to the design and development of more effective and tolerable drugs. Historically, promiscuous drugs with polypharmacological profile have been thought to be more effective for treating CNS disease, although this

represented presence of many severe and potentially life-threating side effects. Therefore, design of selective drugs that would interact with defined molecular targets could probably result in more effective and tolerable drugs. Hence, biased agonism alone or in combination with inverse agonism arise as alternative approaches to receptor selectivity in order to improve the expected functional responses in schizophrenia.

# Aims

Despite much research has been undertaken to develop new antipsychotic drugs for treating effectively the intrinsic impairments, none of the medications currently available has stood out for long-lasting efficacy without detrimental side effects. This is partially due to poorly understood neurobiology of schizophrenia. Therefore, a deeper knowledge of schizophrenia at a neurobiological level would enable the identification of molecular, cellular and/or pathway alterations, which could become therapeutic targets for new drugs.

Several findings suggest that the 5-$HT_{2A}$R is involved in the molecular mechanism responsible of psychotic symptoms and their treatment. On the one side, the 5-$HT_{2A}$R is responsible for the hallucinogenic nature of psychedelic drugs like LSD, psilocybin, mescaline and (±)DOI, which activate the 5-$HT_{2A}$R. On the other hand, atypical antipsychotics commonly used in schizophrenia would act as antagonist or even inverse agonist of the 5-$HT_{2A}$R.

Different studies by *in vivo* PET neuroimaging and *in vitro* post-mortem studies have shown conflicting results about 5-$HT_{2A}$R brain density in schizophrenia subjects. These conflicting reports seem to be related to the use of different radiotracers binding to different conformations of the receptor. Therefore, 5-$HT_{2A}$R alterations in schizophrenia seem to be more related to variations in molecular state of the receptor rather than alterations in expression levels. In this context, a full characterization of 5-$HT_{2A}$R inverse agonists in human brain becomes an unmet need to clarify the functional status of this receptor in schizophrenia.

The 5-$HT_{2A}$R is able to activate both $G\alpha_{q/11}$- and $G\alpha_{i/o}$-proteins depending on the drug binding properties. In this context, activation of $G\alpha_{i/o}$-proteins by 5-$HT_{2A}$R agonist has been proposed as a molecular fingerprint of hallucinogenic properties. Moreover, higher stimulation of $G\alpha_{i1}$- but not $G\alpha_{q/11}$-proteins in response to the 5-$HT_{2A}$R agonist (±)DOI has been described in post-mortem PFC of subjects with schizophrenia. This finding could be interpreted as

enhanced biased agonism of 5-$HT_{2A}$R towards hallucinogenic $G\alpha_{i1}$-protein pathways in schizophrenia. In this sense, the $G\alpha_{i1}$-protein-mediated overactivity could represent the existence of an increased constitutive activity of the 5-$HT_{2A}$R in schizophrenia. In order to clarify whether this enhanced 5-$HT_{2A}$R constitutive activity in native human brain tissue exists, drugs behaving as inverse agonist of the 5-$HT_{2A}$R are necessary tools.

If the previous hypothesis was confirmed, drugs behaving as 5-$HT_{2A}$R inverse agonist on $G\alpha_{i1}$-proteins would be a suitable antipsychotic treatment strategy, independently of their intrinsic activity on $G\alpha_{q/11}$-protein-mediated pathways. Interestingly, several studies suggest that some of the most effective antipsychotics drugs displaying high 5-$HT_{2A}$R affinity are in fact 5-$HT_{2A}$R inverse agonist rather than neutral antagonist. However, the information about pharmacological properties of antipsychotics has been mainly delineated in cell lines, while studies in native tissue are scare. Moreover, all the characterization studies are usually limited to one signalling pathway with no information about the functional selectivity profile of each drug.

In this framework, the first objective of this research work was the characterization of inverse agonist properties of different 5-$HT_{2A}$R drugs previously considered antagonist drugs. By using inverse agonist, the existence of constitutive activity of 5-$HT_{2A}$R coupling to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in post-mortem human brain cortex was tested.

A subsequent objective was to evaluate the possible alteration of the 5-$HT_{2A}$R coupling activity to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in post-mortem human brain of schizophrenia subjects.

Finally, the inverse agonist properties of second-generation antipsychotic drugs on the 5-$HT_{2A}$R coupling to different G-proteins was studied

# Subjects, Materials and Methods

## 3.1 Human brain samples

### 3.1.1 Subjects selection, demographic characteristics, psychiatric diagnosis and toxicological analysis

Human brains were obtained at autopsy in the Basque Country Institute of Legal Medicine, Bilbao, Spain. Samples correspond to subjects dead by suicide, accident, homicide or natural death. Samples were obtained in compliance with research policies and ethical boards for post-mortem human brain studies. The Ethics Board of the University of the Basque Country approved the study (CEISH-UPV/EHU, Ref. M10-2019-230).

After confirmation of death by coroner, the corps were stored at 4°C until autopsy. Samples from the dorsolateral prefrontal cortex (DLPFC) were dissected at the time of autopsy and immediately stored at -80°C until assay. The selection of this brain area was based on previous studies describing it to be implicated in the pathophysiology of schizophrenia (Lewis & Gonzalez-Burgos, 2008; Shepherd et al., 2012; Haber & Robbins, 2022; Kolk & Rakic, 2022).

Acute toxicological screenings on blood (quantitative assays for antidepressants, antipsychotics, other psychotropic drugs, opiates and ethanol) were performed by using standard procedures (radioimmunoassay, enzymatic immunoassay, high-performance liquid chromatography-mass spectrometry) at the National Institute of Toxicology (Madrid, Spain). Nevertheless, since some subjects might have been under antipsychotic treatment in previous periods of the disease, toxicological analysis in brain samples was also performed. Brain tissue accumulates drugs for longer periods than plasma, due to the high content in lipids and, therefore, quantification of antidepressants, antipsychotics, other psychotropic drugs and cotinine was performed in brain samples of the selected subjects. Antiepileptic drugs such as phenobarbital, phenytoine, levetiracetam and valproic acid were

not included in the toxicological study. Analyses were carried out with an Agilent Technologies (Wilmington, DE, USA) 1200 Series HPLC system and 6410 triple Quad mass spectrometer, using MassHunter software and equipped with electrospray ionization (ESI) as the ion source operating in the positive mode. A ZOBRAX (Agilent) Eclipse Plus C8 Narrow Bore (2.1 mm x 150 mm, 5 µm) was selected and the guard column contained the same packing material (Sampedro et al., 2012). These brain toxicological evaluations were performed at the analytical facilities of University of the Basque Country (SGIKER).

Three experimental groups were designed for Antibody-capture [$^{35}$S]GTPγS SPA and Western Blot experiments: schizophrenia subjects, non-schizophrenia suicide subjects and control subjects. All the subjects were matched, whenever possible, for age, gender and post-mortem delay (time interval between death and autopsy, PMD) and storage time. The criteria for diagnosis and cause of death for these groups are explained below:

**Schizophrenia subjects:** Samples included in this group were obtained from subjects that had been diagnosed with schizophrenia according to international criteria (DSM IV, DSM IV-TR) and being excluded an additional psychiatric diagnosis. This group include twenty three schizophrenia subjects (seven female and sixteen male, mean±SEM: age 48±4 years, PMD 22±2 hours, storage time 150±15 months). As determined by the medical examiner, thirteen of the schizophrenia subjects were suicide victims who died from trauma caused by jumping from a height (n=10), suffocation by hanging (n=2) or suffocation by drowning (n=1). The ten schizophrenia subjects who did not commit suicide died from cardiorespiratory failure (n=5), tumour (n=1), haemorrhage (n=1), shock (n=1), accidental choking (n=1) and falling from a height (n=1). All schizophrenia subjects had no presence of antipsychotic drugs in blood according with the toxicological screening at the time of autopsy. (**Table 3.1**).

**Non-schizophrenia suicide subjects:** Subjects ascribed to this group had been diagnosed of personality disorder (n=8), anxiety disorder (n=4) and obsessive-compulsive disorder (n=1), according to DSM criteria, and all of them committed suicide. The study included thirteen non-schizophrenia suicide subjects (one female and twelve male, mean±SEM: age 38±3 years, PMD 16±2 hours, storage time 207±15 months). The suicide subjects died from trauma caused by falling from a height (n=8), suffocation by hanging (n=2), self-inflicted gunshot (n=1), drug overdose (n=1) and suffocation by drowning (n=1). (**Table 3.1**).

**Control subjects:** Brain samples from subjects that had no ante-mortem neurological or psychiatric disorders were included in this group. Twenty three control subjects (seven female and sixteen male, mean±SEM: age 48±3 years, PMD 21±2 hours, storage time 139±15 months). Control subjects died by traffic accident (n=12), cardiorespiratory failure (n=4), accidental falling from a height (n=4), myocardial infraction (n=1), accident by fire (n=1) and trauma caused by falling to the railways (n=1). (**Table 3.1**).

Brain toxicology confirmed the absence or presence of active toxicological concentrations of psychotropic drugs in controls, and antipsychotic drugs in schizophrenia and non-schizophrenia suicide groups (**Table 3.1**).

**Table 3.1:** Demographic characteristics, post-mortem conditions, cause of death and toxicological analysis of individual cases of schizophrenia subjects (**S**), non-schziophrenia suicide (**NSS**) and their respective controls (**C**).

| Case | Diagnostic | Gender (M/F) | Age (years) | PMD(hours) | Storage (months) | Cause of death | Mechanism of death | Brain pH | Drug Blod Levels (mg/L) | Brain Toxicology (ng/g) |
|---|---|---|---|---|---|---|---|---|---|---|
| **S 1** | Schziophrenia | F | 67 | 22 | 17 | Natural | Cardiorespiratory failure | 5.8 | Negative | Negative |
| **C 1** | Control | F | 66 | 17 | 83 | Accident | Road accident | 6.06 | Negative | Not performed |
| **S 2** | Schizophrenia | M | 34 | 23 | 81 | Suicide | Jump from a height | 6.32 | Negative | Cotinine 23.1 |
| **C 2** | Control | M | 34 | 17 | 69 | Accident | Road Accident | 6.7 | Negative | Negative |
| **NSS 1** | Personality disorder | M | 34 | 7 | 199 | Suicide | Hanging | Not performed | Ethanol 2.7 g/L<br>Citalopram 0.1<br>Oxacarbazepina 6.5 | Citalopram 1579.9<br>Cotinine 3352.8<br>Norcitalopram 368.2 |
| **S 3** | Schizophrenia | F | 53 | 18 | 98 | Natural | Haemorrage | 6.58 | Alprazolam 0.05 | Paliperidone 54.2<br>Cotinine 503.2<br>Alprazolam 43.9 |
| **C 3** | Control | F | 51 | 10 | 70 | Natural | Cardiorespiratory failure | 6.3 | Negative | Negative |
| **S 4** | Schizophrenia | M | 32 | 21 | 87 | Suicide | Jump from a height | 6.65 | Negative | Cotinine 380.78 |
| **C 4** | Control | M | 33 | 23 | 95 | Accident | Road accident | 6.55 | Negative | Not performed |
| **NSS 2** | Personality disorder | M | 33 | 14 | 192 | Suicide | Jump from a height | Not Performed | Lorazepam 0.03<br>Venlafaxine 0.16 | Desmethylvenlafaxine 475.0<br>Lorazepam 1421.2<br>Midazolam 3.7<br>Olanzapine 6 |
| **S 5** | Schizophrenia | M | 45 | 36 | 15 | Accident | Choking | Not performed | Not performed | Midazolam 1801.8<br>Nordiazepam 1247.1<br>Oxazepam 50.1 |
| **C 5** | Control | M | 44 | 23 | 98 | Accident | Road accident | 6.45 | Negative | Negative |
| **NSS 3** | Personality disorder | M | 44 | 9 | 192 | Suicide | Jump from a heihgt | Not performed | Ethanol 0.22 g/L<br>Alprazolam 0.01<br>Amisulpride 1.4<br>Clomipramine 0.2<br>Reboxetine 0.09 | Alprazolam 141.4<br>Clomipramine 3040.7<br>Cotinine 322.5<br>Reboxetine 224.2 |
| **S 6** | Schizophrenia | M | 49 | 23 | 107 | Accident | Fall from a height | 6.40 | Negative | Cotinine 391<br>Lorazepam 16.3 |
| **C 6** | Control | M | 49 | 19 | 93 | Accident | Road accident | 6.7 | Negative | Negative |
| **S 7** | Schizophrenia | M | 70 | 20 | 115 | Suicide | Hanging | Not performed | Not performed | Negative |
| **C 7** | Control | M | 71 | 22 | 115 | Accident | Fall from a height | 5.92 | Negative | Negative |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **S 8** | Schizophrenia | F | 74 | 9 | 118 | Natural | Cardiorespiratory failure | Not performed | Phenobarbital 9 | Negative |
| **C 8** | Control | F | 74 | 30 | 167 | Accident | Road accident | Not performed | Negative | Negative |
| **S 9** | Schizophrenia | M | 46 | 20 | 129 | Suicide | Jump from a height | 6.41 | Negative | Zuclopenthixol 110.8<br>Cotinine 622.4<br>Lorazepam 25.5 |
| **C 9** | Control | M | 46 | 22 | 115 | Natural | Fall | 6.48 | Negative | Negative |
| **NSS 4** | Personality disorder | M | 47 | 4 | 195 | Suicide | Jump from a height | Not performed | Phenytoine | Negative |
| **S 10** | Schizophrenia | M | 26 | 24 | 140 | Suicide | Jump from a height | Not performed | Diazepam 0.27 | Diazepam 356.5<br>Nor-diazepam 856.9<br>Oxazepam 15.1 |
| **C 10** | Control | M | 25 | 21 | 71 | Accident | Fire | 6.48 | Negative | Negative |
| **NSS 5** | Personality disorder | M | 27 | 42 | 253 | Suicide | Jump from a height | Not performed | Negative | Cotinine 337.4<br>Diazepam 204.9<br>Nordiazepam 612<br>Oxazepam 65.8 |
| **S 11** | Schizophrenia | F | 75 | 18 | 140 | Natural | Cardiorespiratory failure | Not performed | Negative | Cotinine 36.76 |
| **C 11** | Control | F | 79 | 24 | 213 | Accident | Road accident | Not performed | Negative | Not performed |
| **S 12** | Schizophrenia | M | 28 | 28 | 143 | Suicide | Jump from a heihgt | Not performed | Negative | Cotinine 93.79 |
| **C12** | Control | M | 29 | 13 | 116 | Accident | Fall from a height | 6.44 | Negative | Negative |
| **NSS 6** | Personality disorder | M | 28 | 5 | 259 | Suicide | Jump from a height | Not performed | Negative | Cotinine 638.2 |
| **S 13** | Schizophrenia | M | 25 | 17 | 145 | Suicide | Jump from a height | Not performed | Negative | Cotinine 153.1 |
| **C 13** | Control | M | 23 | 16 | 141 | Accident | Fall from a height | Not performed | Negative | Negative |
| **NSS 7** | Obsessive-compulsive disorder | M | 26 | 19 | 143 | Suicide | Jump from a height | 6.66 | Clomipramine 0.5<br>Fluoxetine 0.7<br>Fluvoxamine 0.2<br>Quetiapine 0.5 | Fluoxetine 15441<br>Fluvoxamine 4094<br>Norfluoxetine 4433.5<br>Norquetiapine 386.7<br>Quetiapine 86 |
| **S 14** | Schizophrenia | M | 23 | 13 | 195 | Suicide | Jump from a height | Not performed | Not performed | Haloperidol 136.5<br>Cotinine 458.5<br>Quetiapine 392.5<br>Norquetiapine 1309.4 |
| **C 14** | Control | M | 22 | 20 | 188 | Accident | Road accident | Not performed | Nordiazepam 0.38 | Cotinine 371.6<br>Oxazepam 80.9<br>Nordiazepam 909 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **NSS 8** | Personality disorder | M | 19 | 8 | 131 | Suicide | Fall from a height | 6.7 | Negative | Cotinine 236.4 |
| **S 15** | Schizophrenia | F | 80 | 32 | 178 | Natural | Shock | Not performed | Negative | Not performed |
| **C 15** | Control | F | 78 | 12 | 185 | Natural | Cardiorespiratory failure | Not performed | Negative | Not performed |
| **S 16** | Schizophrenia | F | 38 | 23 | 216 | Suicide | Jump from a height | Not performed | Negative | Negative |
| **C 16** | Control | F | 36 | 19 | 110 | Accident | Fall in front of a train | 6.51 | Negative | Negative |
| **NSS 9** | Anxiety disorder | F | 39 | 19 | 214 | Suicide | Drug overdose | Not performed | Nordiazepam 0.13<br>Ethanol 5.0 g/L | Diazepam 22.4<br>Nordiazepam 280.7 |
| **S 17** | Schizophrenia | F | 51 | 15 | 236 | Natural | Cardiorespiratory failure | Not performed | Buflomedil 66<br>Metamizol 4 | Cotinine 521.5 |
| **C 17** | Control | F | 51 | 38 | 230 | Accident | Road accident | Not performed | Negative | Not performed |
| **S 18** | Schizophrenia | M | 62 | 28 | 240 | Suicide | Jump from a height | Not performed | Not performed | Amitriptyline 170.3<br>Nortryptiline 453 |
| **C 18** | Control | M | 62 | 23 | 243 | Accident | Road accident | Not performed | Negative | Not Performed |
| **NSS 10** | Adaptative anxiety disorder | M | 62 | 19 | 271 | Suicide | Crushing | Not performed | Not performed | Maprotiline 257.9<br>Nordiazepam 1584.9 |
| **S 19** | Schizophrenia | M | 35 | 22 | 299 | Suicide | Jump from a height | Not performed | Negative | Negative |
| **C 19** | Control | M | 36 | 22 | 286 | Accident | Road accident | Not performed | Ethanol 1.0 g/L | Not performed |
| **NSS 11** | Anxiety disorder | M | 36 | 15 | 181 | Suicide | Gun shot | Not performed | Mirtazapine 0.08 | Not Performed |
| **S 20** | Schizophrenia | M | 49 | 41 | 292 | Suicide | Hanging | Not performed | Ethanol 0.49 g/L/<br>Not performed | Cotinine 127<br>Chlorpromazine 140.7<br>Lorazepam 388.6<br>Thioridazine 6079.5 |
| **C 20** | Control | M | 45 | 30 | 275 | Accident | Road accident | 6.82 | Ethanol 3.09 g/L | Not performed |
| **NSS 12** | Anxiety disorder | M | 46 | 26 | 319 | Suicide | Hanging | Not performed | Not performed | Diazepam 91.3<br>Nordiazepam 175.2 |
| **S 21** | Schizophrenia | F | 56 | 24 | 125 | Natural | Cardiorespiratory failure | Not performed | Alprazolam 0.03 | Cotinine 110<br>Alprazolam 38 |
| **C 21** | Control | F | 54 | 24 | 10 | Accident | Fall from a height | 6.87 | Negative | Negative |
| **S 22** | Schizophrenia | M | 50 | 3 | 173 | Suicide | Drowning | 7.09 | Nordiazepam 0.4 | Amisulpride 75.9<br>Cotinine 89<br>Nordiazepam 662.8<br>Oxazepam 47.6<br>Trazodone 241.6 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **C22** | Control | M | 50 | 2 | 15 | Natural | Cardiorespiratory failure | 6.1 | Negative | Negative |
| **NSS 13** | Personality disorder | M | 49 | 22 | 141 | Suicide | Fall from a height | 6.35 | Nordiazepam 2.8<br>Tiapride 5.4<br>Venlafaxine 1.2 | Cotinine 254.1<br>Desmethylvenlafaxine 265.6<br>Oxazepam 94.7<br>Tiapride 973.5<br>Venlafaxine 2146.5 |
| **S 23** | Schizophrenia | M | 43 | 17 | 172 | Natural | Cardiorespiratory failure | Not performed | Negative | Cotinine 1003.9<br>Lorazepam 66 |
| **C 23** | Control | M | 41 | 15 | 7 | Natural | Cardiorespiratory failure | Not performed | Negative | Negative |

All demographic characteristics, age, PMD, storage time and pH are summarized in **Table 3.2**. As expected, no differences were found when compared age between the three groups (F[2,56]=2.08, p=0.1341). In the same way, no statistical differences between groups were found in either PMD (F[2,56)]=1.99, p=0.1456) or pH values (F[2,21]=0.17, p=0.8433). However, a longer storage time was observed in non-schizophrenia suicide subjects compared to controls (F[2,56]=4.66, p=0.0135) (Table 3.2).

**Table 3.2**: Demographic and post-mortem characteristics of schizophrenia, non-schizophrenia suicide and control subjects included in the study. Group values are means±SEM.

| Group | Gender | Age (years) | PMD | pH | Storage time (months) |
|---|---|---|---|---|---|
| Schizophrenia | 7 F/16 M | 48±4 | 22±2 | 6.5±0.1 | 150±15 |
| Non-schizophrenia suicide | 1 F/12 M | 38±3 | 16±3 | 6.6±0.1 | 207±15* |
| Control | 7 F/16 M | 48±3 | 21±2 | 6.5±0.1 | 139±15 |

*p<0.05 vs control group (Bonferroni's multiple comparison test).

### 3.1.2 Demographic characteristics and diagnosis of subjects included in pools used for Antibody-capture [$^{35}$S]GTPγS Scintillation proximity Assays (SPA) and Western Blot characterization assays

The initial pharmacological characterization of drugs was performed with a pool of DLPFC from different control subjects not included in the rest of the study. In each membrane preparation, homogenates from six prefrontal human brain samples were used. Fifteen samples collected between years 2016 and 2019 were used for the whole study (30% men and 70% women) with a mean age of 59±6 years, PMD of 11±2 hours and storage time of 27±3 months. Absence of toxicological positive test for psychotropic drugs in blood was confirmed in subjects contributing to these pools.

## 3.2 Animals: Transgenic mice

The 5-$HT_{2A}R$ knock-out (5-$HT_{2A}R^{(-/-)}$) and wild-type (5-$HT_{2A}R^{(+/+)}$) mice were generously donated by Prof. R. Maldonado (Barcelona, Spain). Animals had been originally generated on a 129S6/SvEv background further back crossed into the inbred C57BL/6J line, following standard procedures (González-Maeso et al., 2003; Weisstaub et al., 2006; González-Maeso et al., 2007; Orejarena et al., 2011). After donation, supplementary backcrosses were performed. Animals were genotyped by conventional polymerase chain reaction (PCR) and subsequent electrophoresis in our laboratory (data not shown), as described by Fiorica-Howells et al., (Fiorica-Hollowells et al., 2002). Absence of 5-$HT_{2A}R$ expression in 5-$HT_{2A}R^{(-/-)}$ mice was confirmed by the lack of [$^3$H]ketanserin binding (Muguruza et al., 2013) and $G\alpha_{i1}/G\alpha_{q/11}$-protein activation by (±)DOI (Garcia-Bea et al., 2019).

Experiments were performed on adult (15-20 weeks old) C57BL/J6 mice. Animals were housed up to five individuals under standard laboratory conditions (22±1°C, 55±5% relative humidity, 12 h light/dark cycle and free access to standard rodent chow and water). Efforts were made to minimize the number of animal used. The experimental protocols were reviewed and approved by the Local Ethical Committee of Animal Research of the University of the Basque Country (UPV/EHU, CEEA, Ref. M20-2019-321). All experiments were carried out in accordance with the European Community Council Directive on “The Protection of Animals Used for Scientific Purpose” (European Union Directive 2010/63/UE) and Spanish Law (Royal decree 53/2013) for the care of laboratory animals. Adult mice were sacrificed by cervical dislocation, brains removed, cortex dissected and samples stored at -80°C until assay as previously described (Diez-Alarcia et al., 2016).

## 3.3 Drugs

-(±)-DOI: (±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride (Sigma-Aldrich; Saint Louis, Missouri, USA).

-Ketanserin: 3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1*H*,3*H*]-quinazolinedione tartrate (Tocris; Bristol UK).

-MDL100907 (volinanserin): (R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-pipidinemethanol (Sigma-Aldrich; Saint Louis, Missouri, USA).

-Altanserin: 3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone hydrochloride hydrate (Sigma-Aldrich; Saint Louis, Missouri, USA).

-Pimavanserin (ACP-103): 1-(4-Fluorobenzyl)-3-(4-isobutoxybenzyl)-1-(1-methylpiperidin-4-yl)urea (Axon Medchem; Groningen, The Netherlands).

-Nelotanserin: 1-(3-(4-bromo-2-methyl-2H-pyrazol-3-yl)-4-methoxyphenyl)-3-(2,4-difluorophenyl)urea (Axon Medchem; Groningen, The Netherlands).

-Ritanserin: 6-[2-[4-[bis(4-fluorophenyl)methylidene]piperidin-1-yl]ethyl]-7-methyl-[1,3]thiazolo[3,2-a]pyrimidin-5-one (Sigma-Aldrich; Saint Louis, Missouri, USA).

-Eplivanserin: 4-[(*E*,3*Z*)-3-[2-(dimethylamino)ethoxyimino]-3-(2-fluorophenyl)prop-1-enyl]phenol (Axon Medchem; Groningen, The Netherlands).

-MDL-11,939: α-phenyl-1-(2-phenylethyl)-4-piperidinemethanol (Tocris; Bristol, UK).

-SB 242084: 6-Chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1*H*-indole-1-carboxyamide dihydrochloride (Tocris; Bristol, UK).

-Clozapine: 8-Chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine (Tocris; Bristol, UK).

-Risperidone: 3-[2-[4-(6-fluoro-1,2- benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido[1,2- a]pyrimidin-4-one (Sigma-Aldrich; Saint Louis, Missouri, USA).

-Aripiprazole: 7-{4-[4-(2,3-Dichlorophenyl)-1-piperazinyl]butoxy}-3,4-dihydro-2(1H)-quinolinone one (Sigma-Aldrich; Saint Louis, Missouri, USA).
-Olanzapine: 2-Methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5]benzodiazepine (Sigma-Aldrich; Saint Louis, Missouri, USA).
-Paliperidone: 3-[2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-9-hydroxy-2-methyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (Tocris; Bristol, UK).
-Quetiapine: 2-[2-(4-Dibenzo[*b*,*f*][1,4]thiazepin-11-yl-1-piperazinyl)ethoxy]ethanol hemifumarate (Tocris; Bristol, UK).
-Atropine: Endo-(±)-α-(Hydroxymethyl)benzeneacetic acid 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester (Sigma-Aldrich; Saint Louis, Missouri, USA).
-Phentolamine: 2-[N-(3-Hydroxyphenyl)-p-toluidinomethyl]-2-imidazolidine hydrochloride (Sigma-Aldrich; Saint Louis, Missouri, USA).
-Cetirizine: [2-[4-[(4-Chlorophenyl)phenylmethyl]-1-piperazinyl]ethoxy]acetic acid dihydrochloride (Sigma-Aldrich; Saint Louis, Missouri, USA).
-Raclopride: 3,5-Dichloro-N-[[(2S)-1-ethyl-2-pyrrolidinyl]methyl]-2-hydroxy-6-methoxybenzamide (Tocris; Bristol UK).
-Haloperidol: 4-[4-(4-Chlorophenyl)-4-hydroxypiperidino]-4′-fluorobutyrophenone (Sigma-Aldrich; Saint Louis, Missouri, USA).

## 3.4 Materials

### Antibodies

Primary monoclonal antibodies used for Antibody-capture [$^{35}$S]GTPγS SPA and Western Blot experiments were purchased from Santa Cruz Biotechnology, Inc (California USA). They are detailed in **section 3.5.2**, where Western Blot experiment protocol is explained.

For Western Blot assays, different fluorescent secondary antibodies were used, such as Alexa Fluor® 680 conjugated with goat anti-mouse, provided by Invitrogen (Oregon, USA), and IRDye™ conjugated with anti-rabbit, provided by Rockland Immunochemical (Pennsylvania, USA).

### Radioactive compounds

Sulfur 35 labelled guanosine-5'-*O*-(gamma-thio)-triphosphate ([$^{35}$S]GTPγS) with specific activity of 1250 Ci/mmol was from PerkinElmer (Waltman, MA, USA).

### Other drugs and chemicals sources

-Bio-Rad Laboratories (California, USA): Ammonium persulfate (APS), Bradford Protein Assay, 2x concentrated Laemmli sample buffer, N-N-N-N´-tetramethylethylenediamine (TEMED), pre-stained SDS-PAGE molecular weight standards.

-Carlo Erba Reagents (Barcelona, Spain): Methanol.

-GE Healthcare (Buckinghamsire, UK): Nitrocellulose membranes (pore size: 0.45 µm) and Whatman™ cellulose 3 mm.

-Invitrogen (Barcelona, Spain): DL-Dithiothreitol (DTT), ethylenediamine tetracetic acid (EDTA).

-National diagnostics (Atlanta, GA, USA): Acrylamide 30%-bisacrylamide 0.8%.

-Panreac S.A.U (Barcelona, Spain): Glacial acetic acid, sucrose and HCL (37%).

-Sigma-Aldrich® (Saint Louis, Missouri, USA): Bovine serum albumin (BSA), 2-butanol, dimethylsulfoxide (DMSO), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), glycine, guanosine diphosphate (GDP), guanosine 5'-O-[gamma-thio]triphosphate (GTPγS), Igepal® CO-520, β-mercaptoethanol, $MgCl_2$, NaCl, NaF, $Na_3VO_4$, Protease Inhibitor Cocktail, polyoxyethylene (20) sorbitan monolaurate (Tween™ 20), sodium deoxycholate (SDC), sodium dodecyl sulfate (SDS), Tris (hydroxymethyl)aminomethane hydrochloride (Tris HCl)

-Perkin Elmer (Waltham, MA, USA): 96 well isoplates and polyvinyltoluene (PVT) SPA beads coated with protein A.

## 3.5 Methods

### 3.5.1 Antibody-capture [$^{35}$S]GTPγS Scincillation Proximity Assay (SPA)

Receptor-mediated specific activation of different Gα-proteins was determined using a homogeneous protocol of [$^{35}$S]GTPγS binding coupled to immunoprecipitation with specific antibodies known as Antibody-capture [$^{35}$S]GTPγS Scintillation Proximity Assay (SPA) (**Figure 3.1**). This assay measures the ability of a drug to induce a response mediated by a Gα-protein subtype, by determining the changes in the binding of the poorly hydrolysable GTP analog [$^{35}$S]GTPγS to the Gα-subunit. It represents a functional assay that could be used to detect and quantify the efficacy and potency of a drug in a certain system. The maximal effect exerted by an agonist or inverse agonist $E_{max}$/$I_{max}$, which corresponds to the magnitude of maximum asymptote can be calculated. Measurement of potency in this assay corresponds to $EC_{50}$/$IC_{50}$ that represents the concentration of an agonist or inverse agonist required to produce 50% of the maximal stimulatory or inhibitory response, respectively.

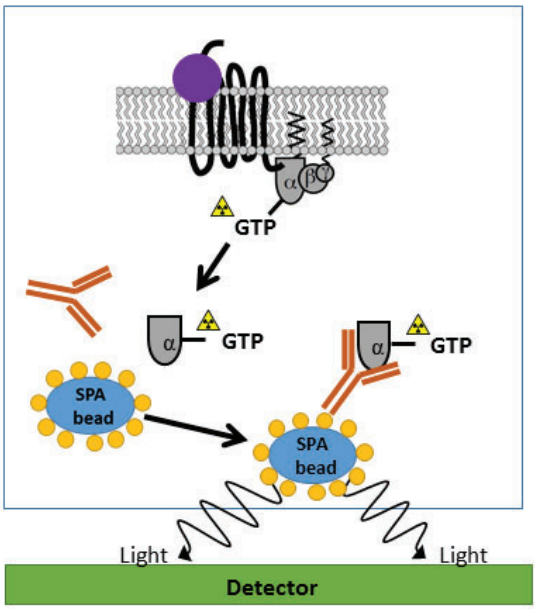



**Figure 3.1:** Schematic representation of Antibody-capture [$^{35}$S]GTPγS Scintillation Proximity Assay (SPA). This figure depicts a single well of 96-well plate containing a membrane preparation bearing as example of an agonist-activated GPCR. The activated Gα subunit, released from the membrane after initial incubation with detergent, binds [$^{35}$S]GTPγS and is recognized by the primary anti-Gα antibody shown in grey. The measurement or radioactive energy is only limited to radioactive molecules that are held in close proximity to a polymer microsphere containing scintillant (SPA bead). The beads capture antibody-Gα protein complex. This interaction allows conversion of radioactivity to detectable photon emission by the scintillant and that is read by a microplate scintillation counter. *Illustration originally created for this thesis by C. Muguruza and adapted from DeLapp NW. 2004*.

### 3.5.1.1 Preparation of membrane-enriched fraction ($P_2$ fraction)

Membrane preparations were performed as previously reported in detail (González-Maeso et al., 2000) with minor modifications. Brain cortex samples (approximately 1 g from human and 200 mg from mouse) from each subject or animal were thawed slowly at 4°C, and homogenized using a Teflon-glass grinder (10 up-and-down strokes at 15000 rpm) in 30 volumes of homogenization buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$ and 1 mM DTT, pH 7.4; supplemented with 250 mM sucrose). The homogenates were centrifuged at 1000 x g (Sorvall RC-5C centrifuge, SM-24 rotor;

FisherScientific, Madrid, Spain) for 5 minutes (4°C). Pellets were discarded and supernatant was centrifuged for 10 minutes at 40,000 x g (4°C). The resultant pellet was resuspended in 20 volume of centrifugation buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$ and 1 mM DTT; pH 7.4), and centrifuged in similar conditions. The obtained pellets were then resuspended in five volumes of centrifugation buffer. Aliquots of 0.5 mg were then centrifuged at 21,000 x g (Eppendorf 5810R centrifuge, Eppendorf, Madrid, Spain) during 15 minutes (4°C). The supernatant layer was carefully discarded and the pellets were stored at -80°C until assay.

Protein content was measured according to the Bradford method (1976) using BSA as standard (González-Maeso et al., 2000). Briefly, 200 µl of Bradford Protein assay reagent, diluted in 1:5 MilliQ water, was added to 10 µl protein suspension (diluted 1:3 and 1:4). After incubation at room temperature for 6 minutes, absorbance at 630 nm wavelength was determined in an ELX808 microplate reader. Protein values were extrapolated from standard line performed with BSA from 0 to 0.5 mg/ml.

### 3.5.1.2 Antibody-capture [$^{35}$S]GTPγS Scincillation Proximity Assay (SPA)

Specific activation of different Gα-proteins by the 5-$HT_{2A}$R was determined by using a protocol of Antibody-capture Scintillation Proximity Assay (SPA), which is based on a [$^{35}$S]GTPγS binding assay combined with specific antibodies. Assays were performed as previously described with minor changes (Erdozain et al., 2012; Diez-Alarcial et al., 2016; Moreno et al., 2016; Ibarra-Lecue et al., 2018; Garcia-Bea et al., 2019; Muneta-Arrate et al., 2020; Brocos-Mosquera et al., 2021; Diez-Alarcia et al., 2021b).

First, membrane-enriched fractions were slowly thawed at 4°C, and resuspended in SPA incubation buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT and 100 mM NaCl; pH 7.4), reaching a final protein

concentration of 68,75 µg/ml. [$^{35}$S]GTPγS binding was performed in 96-well isoplates. Final volume of 200 µl/well contained SPA vehicle (SPA incubation buffer/ DMSO), 0.4 nM [$^{35}$S]GTPγS (1250 Ci/mmol), 11 µg of protein, and different concentrations of GDP (100-50 µM), depending on the Gα-protein subunit (**Table 3.3**). For non-specific binding measurement (NSB), an excess of unlabelled GTPγS (100 µM) was added (**Table 3.4**).

**Table 3.3:** GDP concentrations and antibody dilutions employed in the antibody-captures [$^{35}$S]GTPγS SPA assay.

| Target | [GDP] | Antibody dilution |
|---|---|---|
| $G\alpha_{i1}$ | 100 µM | 1:20 |
| $G\alpha_{i2}$ | 50 µM | 1:20 |
| $G\alpha_{i3}$ | 100 µM | 1:60 |
| $G\alpha_{o}$ | 50 µM | 1:40 |
| $G\alpha_{q/11}$ | 50 µM | 1:40 |

Brain membranes were preincubated for 30 minutes at 30°C in presence of the drugs of interest, in order to ensure that all drugs were at equilibrium during the labelling period. After 90 minutes of incubation at 30°C with gentle agitation (400 rpm), 20 µl of detergent solution (1% Igepal, 0.1% sodium deoxycholate, 1% protease inhibitor cocktail) was added to each well to solubilize membranes without destroying the [$^{35}$S]GTPγS-Gα association, and to avoid the effect of proteases that could reduce the signal over time (Harder & Fotiadis, 2013). After solubilisation, 10 µl of diluted selective antibodies for each Gα subunit **(Table 3.3)** were added to each well. Antibodies were pre-diluted in 15% BSA at different concentrations for each Gα-protein subtype (Diez-Alarcia et al., 2021b). Finally, after an incubation period of 90 minutes, 50 µl of PVT protein A SPA beads at 0.75 mg/ml concentration were added, and plates were incubated for 180 minutes at 22°C with gentle agitation (350 rpm) (**Table 3.4**). The incubation allows protein A coating beads to interact with

antibodies previously bound to the different Gα subtype (Ferrer et al., 2003). Plates were centrifuged (15 minutes at 1,000 x g) before quantification using a MicroBeta TriLux scintillation counter (PerkinElmer España, S.L., Madrid, Spain).

**Table 3.4:** General protocol of [$^{35}$S]GTPγS binding combined with SPA. **BB**: Basal binding (binding values in absence of any drug), **NBS**: Non-specific binding (defined as the remaining [$^{35}$S]GTPγS binding in presence of 100 μM unlabelled GTPγS).

| | BB | Agonist | Agonist + Antagonist | NBS |
|---|---|---|---|---|
| **SPA incubation buffer/DMSO** | 24 μl | 12 μl | / | 12 μl |
| **GTPγS** | / | / | / | 12 μL |
| **GDP** | 12 μl | 12 μl | 12 μl | 12 μl |
| **Antagonist** | / | / | 12 μl | / |
| **Membranes** | 160 μl | 160 μl | 160 μl | 160 μl |
| 15 minutes of incubation at 30°C with gentle agitation (400 rpm) | | | | |
| **Agonist** | / | 12 μl | 12 μl | / |
| 30 minutes of incubation at 30°C with gentle agitation (400 rpm) | | | | |
| **[$^{35}$S]GTPγS** | 5 μl | 5 μl | 5 μl | 5 μl |
| 90 minutes of incubation at 30°C with gentle agitation (400 rpm) | | | | |
| **Detergent** | 20 μl | 20 μl | 20 μl | 20 μl |
| 30 minutes of incubation at 22°C with gentle agitation (350 rpm) | | | | |
| **Antibodies** | 10 μl | 10 μl | 10 μl | 10 μl |
| 90 minutes of incubation at 22°C with gentle agitation (350 rpm) | | | | |
| **PVT Protein A SPA BEADS** | 50 μl | 50 μl | 50 μl | 50 μl |
| 180 minutes of incubation at 22°C with gentle agitation (350 rpm) | | | | |

In general, in order to find a selective inverse agonist, initially, different drugs previously described as 5-HT$_{2A}$R antagonist were chosen and tested for inverse agonism properties. After that, selected inverse agonists compounds were used to investigate 5-HT$_{2A}$R alterations coupling to Gα-proteins in schizophrenia subjects. In an effort to evaluate the specificity and selectivity of these drugs, different selective antagonist as well as 5-HT$_{2A}$R$^{(+/+)}$ and 5-HT$_{2A}$R$^{(-/-)}$ mice membrane homogenates were used to block the [$^{35}$S]GTPγS binding signal observed for each drug.

### 3.5.1.3 Mathematical and statistical analysis of the results

#### Mathematical analysis

Results obtained from the Microbeta Trilux Scintillation counter are expressed as CCPM (corrected counts per minute), which assumes the equivalency between CCPM and DPM (disintegrations counts per minute). In order to interpret the results, data from CCPM must be transformed into femtomole [$^{35}$S]GTPγS bound per milligram protein (fmol/mg protein). The conversion is made considering the protein concentration used in the assay and by following the **Equation 3.1.** The protein concentration can influence the [$^{35}$S]GTPγS binding as shown in Diez-Alarcia et al., 2021b. Therefore, protein content of the assay is measured after each experiment.

**fmol/ mg protein**: CCPM counts/(2,22 x 1250 x [assay protein concentration mg])

**Equation 3.1**: Results expressed in fmol/mg protein performed in [$^{35}$S]GTPγS binding combined with SPA. 1250 (Ci/mmol) corresponds to specific activity of the radioligand $^{35}$S. 2,22 is a constant for the transformation from Curie (Ci) to DMP (1 Ci=2,22x10$^{12}$ DPM).

Basal binding (BB) of [$^{35}$S]GTPγS, is defined as [$^{35}$S]GTPγS binding in absence of any exogenous drug. When an agonist or inverse agonist drugs is added to the assay the basal [$^{35}$S]GTPγS binding is modulated. When the drug is an agonist, an increase of [$^{35}$S]GTPγS binding on [$^{35}$S]GTPγS basal binding will be observed. In contrast, inverse agonists will decrease the [$^{35}$S]GTPγS basal binding.

The non-specific binding (NBS) in CCPM was subtracted from all CCPM obtained for each different condition (basal binding, stimulation, inhibition,..). Thus, specific [$^{35}$S]GTPγS binding values were obtained and used for further calculation and statistical analysis.

Concentration-response curves for different drugs were performed in order to determine agonist, antagonist or inverse agonist properties of each drug. The BB, in absence of the drug, was considered as 100%, and specific binding were transformed to percentage of basal binding. Thus, stimulation or inhibition effect on the [$^{35}$S]GTPγS binding in presence of the drug was defined in relation to respective basal binding. Each point of the curve was calculate with **Equation 3.2** and **3.3.**

| % Stimulation: (Stimulation-NBS)/(BB-NBS)x100 |
|---|

**Equation 3.2**: Calculation of percentage over basal binding performed in [$^{35}$S]GTPγS binding combined with SPA experiments in order to determine agonist, antagonist or competition effects. **NBS**: Non-specific binding, **BB**: Basal binding.

| % Inhibition: 100-[(Inhibition-NBS)/(BB-NBS)x100] |
|---|

**Equation 3.3**: Calculation of percentage of basal binding performed in [$^{35}$S]GTPγS binding combined with SPA experiments in order to determine inverse agonist, antagonist or competition effects. **NBS**: Non-specific binding. **BB**: Basal binding.

In some cases, in order to test the effect of [$^{35}$S]GTPγS binding to different Gα-protein subtypes, instead of concentration-response curves, a single concentration (10 μM) of the drug was used. This concentration was selected because it gives binding values around the maximal effects ($E_{max}$/$I_{max}$) in the concentration-response curves. This concentration was selected for one-point concentration experiments. In this case, basal binding was defined as 0% and effects were expressed as positive or negative percentage changes respect to basal conditions

Pharmacological parameters of stimulation or inhibition curves of [$^{35}$S]GTPγS binding, maximal stimulatory or inhibitory effects ($E_{max}$/$I_{max}$) and concentration

of the drug that determines half maximal stimulation or inhibition ($EC_{50}/IC_{50}$) were calculated. These parameters were obtained by non-linear analysis using GraphPad Prim™. Points were fitted to the concentration-response curve shown in **Equation 3.4** and **3.5**. This model assumes that concentration-response curve displays a standard slope, equal to a Hill slope (or slope factor) of 1.0. This is the expected slope when a ligand binds to a single receptor according to the law of mass action.

$$E= BB+(E_{max}-BB)(1+10^{(LogEC_{50}-Log[X])})$$

**Equation 3.4**: Monophasic stimulatory concentration-response curve (standard slope). **E** corresponds to the effect (% Stimulation of Equation 3.2) at the **X** concentration, **BB** corresponds to the specific [$^{35}$S]GTPγS binding in absence of agonist or basal binding (assumed as 100%), $\mathbf{E_{max}}$ to the maximal effect (%) and $\mathbf{LogEC_{50}}$ to the concentration of the drug that determines half maximal effect.

$$E= BB+(I_{max}-BB)(1+10^{(LogIC_{50}-Log[X])})$$

**Equation 3.5**: Monophasic inhibitory concentration-response curve (standard slope). **E** corresponds to the effect (% Inhibition of Equation 3.3) at **X** concentration, **BB** corresponds to the specific [$^{35}$S]GTPγS binding in absence of inverse agonist or basal binding (assumed as 100%), $\mathbf{I_{max}}$ to the maximal effect (%) and $\mathbf{LogIC_{50}}$ to the concentration of the drug that determines half maximal effect.

Pharmacological parameters $E_{max}/I_{max}$ are expressed as mean±SEM (standard error of the mean). $-LogEC_{50}/IC_{50}$ are expressed as mean±SEM. These values are used for further statistical analysis. Half-maximal stimulation or inhibition are also expressed as $EC_{50}/IC_{50}$ that corresponds to the antilogarithm of $LogEC_{50}/IC_{50}$ mean values.

Concentration-response curves were simultaneously co-analysed (all the experiments of each experimental group together) by non-linear regression for

the best fit assuming an one-site model of [$^{35}$S]GTPγS binding. This way, global values for each group were obtained. Results are expressed as the best fit value±95% confidence interval.

**Statistical analysis**

Different statistical analyses were used for the suitable interpretation of the results.

Initially, all data were subjected to Grubb´s test, in order to detect and reject possible outlier values among experimental groups. Whether obtained data displayed a Gaussian distribution was also tested.

Results from one single-concentration experiments were analyzed by one-sample Student's t-test vs basal values, in order to evaluate presence or absence of the effect induced by drugs on basal binding.

Two-tailed unpaired Student´s t-test was used to compare two different conditions. For example, to compare the effect of an agonist alone vs agonist co-incubated with an antagonist. When three different conditions comparison was needed, one-way ANOVA analysis was performed followed by Bonferroni´s post-hoc analysis. This test was used, for example, to compare the modulation of [$^{35}$S]GTPγS binding between schizophrenia, non-schizophrenia suicide and control groups.

In order to calculate the contribution effect of two different variables, two-way ANOVA was used followed by Bonferroni´s post-hoc analysis. This analysis was appropriate to study the effect of different drugs in various mice genotypes.

Pearson´s correlation r coefficient was calculated to test possible associations between independent covariables (age, PMD and storage time) and dependent functional responses. When correlation was significant, analysis of

covariance (ANCOVA) was performed between groups (schizophrenia, non-schizophrenia suicide and control subjects) controlling for the independent covariable. ANCOVA analyses were carried out by using InVivoStat statistical software.

In all statistical evaluations, differences were considered significant at $p<0.05$.

A complementary analysis of potential differences between groups was also performed. Thus, global results obtained from coanalysis of schizophrenia, non-schizophrenia suicide and control subjects data were subjected to further evaluation (DeLean et al., 1978; Motulsky & Ransnas, 1987). This extra analysis compared the goodness of fit of a model with and without a set of constrains by means of an F test (based on the principle of extra sum of squares). First, sets of data were analysed separately (no constrains) as described above. Overall value for the sum of squares was sum of individual values from each fit and, similarly, the numbers of degrees of freedom. Next, sets were pooled, analysed simultaneously, and constrained to share one or more common parameters (basal, $E_{max}/I_{max}$ and $LogEC_{50}/IC_{50}$), which gave different values for the sum of squares and degrees of freedom. Analysis that permitted one or more parameters sharing, without a significant increase in the residual variance, was taken as the best fit. Statistical significance of the improvement was determined with a F test at $p<0.05$, and was expressed as F[DFn, DFd] where F is distribution value, DFn are degrees of freedom in numerator and DFd are degrees of freedom in denominator.

### 3.5.2 Western Blot experiments

Western Blot is a broadly used technique for detection and identification of proteins by means of antibodies. The process is divided into various steps: electrophoresis separation by molecular weight of proteins present in the sample; protein transfer from the gel to nitrocellulose membranes, which immobilizes proteins and make them accessible to antibodies; and exposition of nitrocellulose membranes to solution containing the antibodies that recognize and bind specifically a target protein.

In the present study, firstly, Western Blot experiments were performed in order to characterize and evaluate the specificity of antibodies used in antibody-capture [$^{35}$S]GTPγS Scintillation Proximity Assays (SPA) for each Gα-protein subtype. Further, immunoreactive densities of $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins were quantified in post-mortem PFC of schizophrenia, non-schizophrenia suicide subjects and their matched control subjects. Simultaneously, density of cytoskeletal protein β-actin was also measured, as a loading control.

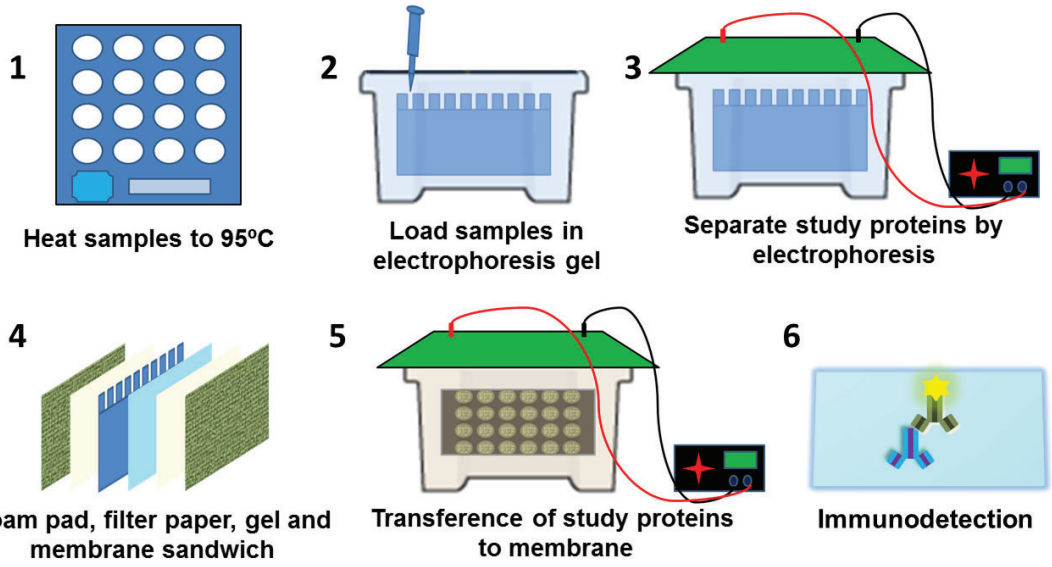



**Figure 3.2**: Schematic representation of Western Blot assays. **1.** Heating of samples at 95°C for five minutes; **2.** Loading of an optimized quantity of sample onto gel; **3.** Afterwards, running of gel electrophoresis at 60 V for 30 minutes and change to 140 V for 90 minutes. Proteins separate according to size, with smaller proteins migrating through the gel; **4.** Assembling of transfer sandwich with the gel near the cathode (-) and the membrane near the anode (+); **5.** Negatively charged proteins will migrate out of the gel onto the membrane by performing transfer at 0.3 A per tray, for 90 minutes; **6.** Blocking the membrane in an appropriate blocking buffer (milk). Incubation of the membrane with primary antibody and, after washing, incubation with secondary antibody for 1 hour at room temperature. Finally, detection of image fluorescence. *Illustration originally created for this thesis by J. DelaCuesta-Barrutia.*

### 3.5.2.1 Preparation of membrane enriched fraction ($P_2$ fraction)

Membrane $P_2$ fractions to perform Western-blot assays were prepared as previously described for SPA assays (**Section 3.5.1.1**). The day of experiment, 0.5 mg of $P_2$ fraction pellets were defrosted and resuspended in 125 µl of Tris-HCl 0.5 nM. These samples were combined with 119 µl 2X Laemmli sample buffer and 6 µl of β-mercaptoethanol, obtaining 2 mg/ml as a final protein content. Samples from schizophrenia, non-schizophrenia suicide and control subjects were processed in parallel on the same day.

### 3.5.2.2 Gel electrophoresis, transference and immunodetection

**Electrophoresis in polyacrylamide gels**

The electrophoresis in polyacrylamide gels in denaturalizing conditions or SDS-PAGE (Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis) is the most used analytical method to separate different proteins of a sample according to their molecular weight.

During the electrophoresis, proteins migrate in first place though “stacking gel”. Stacking gel was prepared with 5% of polyacrylamide, 125 mM Tris HCl, 0.1% SDS, 0.07% prostatic specific antigen (PSA) and 0.14 TEMED pH 6.8. Laemmli-prepared samples were heated at 95°C for five minutes in a Thermoblock during the preparation of “stacking gel” (**Figure 3.2**). Protein separation according to their molecular weight started in the “running gel”. This gel was prepared in a 10% acrylamide-bisacrylamide concentration in a solution of 0.37 M Tris HCl, 0.1% SDS, 0.07% PSA and 0.07% TEMED, pH 8.8. A comb from Teflon™ was inserted in each stacking gel in order to form 15 lanes, where the samples run.

Samples were loaded on those 15-lane gels, sized 6 x 8 cm each (**Figure 3.3**). The loading protein and volume of samples in each line depended on the Gα-protein of interest (**Table 3.5**). The first line of each gel was loaded with a commercial molecular weight marker suspension (10-250 kDa, Precision Plus Protein™, Dual Color Standards, Bio-Rad). The rest of lines were loaded with corresponding volume of the sample. Gel loading sketch is shown in **Figure 3.3**.

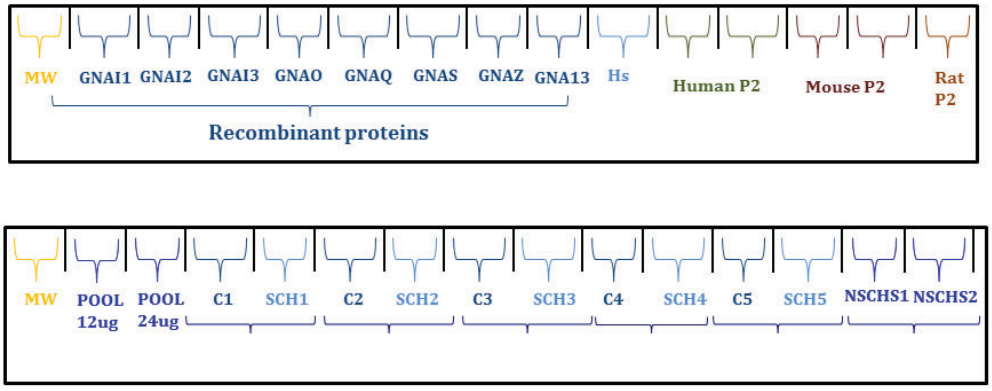



**Figure 3.3**: Gel loading sketch of a western blot experiment for antibody characterization and schizophrenia, non-schizophrenia suicide and control subjects experiments. **MW**: Molecular weight marker, **P2**: membrane-enriched fraction, **C**: Control subjects, **SCH**: Schizophrenia subjects, **NSCHS**: Non-schizophrenia suicide subjects.

**Table 3.5**: Experimental conditions for Western Blot experiments with human, rat and mouse brain samples. **MW** (Molecular weight).

| Protein | Loaded protein/volume | MW (kDa) | Blocking solution | Incubation solution | Primary antibody dilution | Secondary antibody dilution |
|---|---|---|---|---|---|---|
| $G\alpha_{i1}$ | 30 µg/ 15 µl | 40 | 5% nonfat dry milk | Blocking solution + 0.1% Tween 20 | 1:200 | 1:10000 |
| $G\alpha_{i2}$ | 15 µg/ 7.5 µl | 42 | 5% nonfat dry milk | Blocking solution + 0.1% Tween 20 | 1:300 | 1:8000 |
| $G\alpha_{i3}$ | 15 µg/ 7.5 µl | 40 | 5% nonfat dry milk | Blocking solution + 0.1% Tween 20 | 1:300 | 1:8000 |
| $G\alpha_{o}$ | 15 µg/ 7.5 µl | 45 | 5% nonfat dry milk | Blocking solution + 0.1% Tween 20 | 1:500 | 1:8000 |
| $G\alpha_{q/11}$ | 24 µg/ 12 µl | 40 | 5% nonfat dry milk | Blocking solution + 0.1% Tween 20 | 1:300 | 1:8000 |
| $G\alpha_{s}$ | 15 µg/ 7.5 µl | 41 | 5% nonfat dry milk | Blocking solution + 0.1% Tween 20 | 1:500 | 1:8000 |

The electrophoresis was carried out in a buffer consisting of 25 mM Tris HCl, 192 mM glycine and 0.1% SDS pH 8.3. Initially and during 30 minutes, approximately, a 60 V tension was applied, followed by a 140 V tension during running on "running gel" (**Figure 3.2**). Running was stopped when electrophoresis front reached the end of the gel, approximately 90 minutes later.

### Transference to nitrocellulose membranes

Next step was the transference, where proteins were transferred from the gel to a nitrocellulose membrane (pore size 0.45 µM) in a transfer buffer consisted of 25 mM Tris HCl, 192 mM glycine, 20% methanol, pH 8.3. An electric field of 0.3 A per tray was applied during 90 minutes. Gel and nitrocellulose membrane were kept tightly packed with cellulose papers into a cassette, and transference was carried out on ice (**Figure 3.2**).

### Blocking and immunodetection

Once transference was finished, in order to wash from methanol excess, nitrocellulose membranes were rinsed in phosphate buffer saline solution (PBS) (137 mM, NaCl 2.7 mM KCl, 12 mM $Na_2HPO_4$, 1.38 mM $KH_2PO_4$, pH 7.4). Afterwards, nitrocellulose membranes were incubated for 1 hour at room temperature in a blocking solution (5% nonfat dry milk, pH 7.4 in PBS) to eliminate non-specific binding of antibodies and, thereby, reduce the background signal. Then, nitrocellulose membranes were incubated overnight at 4°C in incubation buffer (5 % nonfat dry milk, 0.1% Tween 20 in PBS) containing appropriate dilution of primary antibody (**Table 3.5**). Anti β-actin antibody (rabbit) was also added to the primary antibody solution in a dilution of 1:10000.

Next day, membranes were washed with PBS. Subsequently, fluorescent conjugated secondary antibodies (Alexa Fluor® anti-mouse and IRDye™ anti-rabbit) were added suitably diluted (**Table 3.5**) on incubation buffer for 1 hour at room temperature and constant agitation. Finally, nitrocellulose membranes were washed from an excess of secondary antibodies with PBS.

Nitrocellulose membranes incubated with fluorescent conjugated secondary antibodies were detected and quantified using Odyssey infrared imaging system (LI-COR Bioescience, Nebraska, USA) (**Figure 3.2**). The image of IRDye™ 800 conjugated antibody was captured at 800 nm, visualized in green, while Alexa Fluor® 680 conjugated antibody image was detected at 680 nm, and visualized in red.

For antibody specificity Western Blot, experiments were performed using specific antibodies against Gα-subtypes (**Table 3.6**). Corresponding recombinant proteins (**Table 3.7**) were used to determine the possible cross reactivity among Gα subtypes when using human, mice or rat membrane enriched fractions

**Table 3.6**: Monoclonal Antibodies used in Wenstern Blot and Antibody-capture [$^{35}$S]GTPγS SPA experiments

| Target | Description | Product number | Distributor | M.W | Species reactivity | IgG Subtype | Final concentration |
|---|---|---|---|---|---|---|---|
| $G\alpha_{i1}$ | Mouse monoclonal anti-$G\alpha_{i1}$ | sc-56536 | Santa Cruz | 41 kDa | Ms, R, H | Ms IgG2b | 0.1 µg/well<br>9 ng/µg prot |
| $G\alpha_{i2}$ | Mouse monoclonal anti-$G\alpha_{i2}$ | sc-13534 | Santa Cruz | 41 kDa | Ms, R, H | Ms IgG2b | 0.1 µg/well<br>9 ng/µg prot |
| $G\alpha_{i3}$ | Mouse monoclonal anti-$G\alpha_{i3}$ | sc-365422 | Santa Cruz | 45 kDa | Ms, R, H | Ms IgG3 | 0.033 µg/well<br>3 ng/µg prot |
| $G\alpha_{o}$ | Mouse monoclonal anti-$G\alpha_{o}$ | sc-393874 | Santa Cruz | 40 kDa | Ms, R, H | Ms IgG2a | 0.05 µg/well<br>4.5 ng/µg prot |
| $G\alpha_{q/11}$ | Mouse monoclonal anti-$G\alpha_{q/11}$ | sc-515689 | Santa Cruz | 45 kDa | Ms, R, H | Ms IgG2b | 0.1 µg/well<br>9 ng/µg prot |
| $G\alpha_{s/olf}$ | Mouse monoclonal anti-$G\alpha_{s/olf}$ | sc-377435 | Santa Cruz | 45 kDa/<br>52kDa | Ms, R, H | Ms IgG2a | 0.1 µg/well<br>9 ng/µg prot |

**Table 3.7**: Recombinant proteins used in Western Blot experiments

| Target | Product number | Distributor | M.W | Loaded protein | Batch |
|---|---|---|---|---|---|
| GNAi1 | 32-3896 | Abeomics | 42.7 kDa + 23 aa HisTag | 100 ng | 413PGANI1 |
| GNAi2 | ABIN1355337 | Antibodies on-line | 42 kDa + GST Tag | 50 ng | H2141 |
| GNAi3 | 32-3898 | Abeomics | 43 kDa + 23 aa HisTag | 100 ng | 1114PGANI3 |
| GNAO1 | ABIN5709596 | Antibodies on-line | 40 kDa + 44 aa HisTag | 75 ng | 04055 |
| GNAq | ABIN1355345 | Antibodies on-line | 42 kDa + GST Tag | 75 ng | IC051 |
| GNAs | ABIN1355349 | Antibodies on-line | 47 kDa + GST Tag | 75 ng | HC141 |
| GNAz | CSB-EP009601HU | Cusabio | 56.8 kDa + 6xHis-SUMO | 75 ng | 03251 |
| GNA13 | CSB-EP618885HU | Cusabio | 48 kDa + 6xHisTag | 75 ng | 03257 |

### 3.5.2.3 Mathematical and statistical analysis of results

#### Mathematical analysis

Fluorescence signal of nitrocellulose membranes incubated with fluorescent conjugated antibodies was detected and quantified using Odyssey infrared imaging system (LI-COR, Bioesciences, Nebraska, USA). Integrated intensity values were obtained. The data obtained with Western Blot is considered semi-quantitative because it provides a relative comparison protein level between samples in membranes, but not an absolute measure quantity, due to an standard protein concentration is absent.

In the study, immunoreactivity values of target proteins were corrected for β-actin immunoreactivity, in order to control for loading charge in each lane. β-actin is a cytoskeletal protein which is not altered in brain cortex of schizophrenia or suicide subjects (Urigüen et al., 2009; Rivero et al., 2015). In order to prevent interexperimental variability, estimated relative amount of the target protein was also corrected as percentage of value obtained for the pool sample loaded on the same gel. Pool sample was the same for all Western Blot experiments and was considered as the 100% value. All the samples in each assay were corrected as relative immunoreactivity (in %) respect to integrity signal of the pool in same assay.

The final value for each subject was the mean value of a minimum of three experiments performed in separate gels and days. Results were expressed as mean±SEM values.

**Statistical analysis**

No comparisons were conducted in the experiments performed to characterize bands immunoreactivity because the aim was just to check specificity of the antibodies. Hence, only the presence of a clear single band in appropriate molecular weight level was analysed.

All data were subjected to Grubb´s test in order to detect and reject possible outlier values among experimental groups. Whether the obtained data displayed a Gaussian distribution was also tested.

One-sample Student's t-test vs 100% values was performed in control group in order to confirm the absence of differences in immunoreactivity respect to the pool results.

Immunoreactivity of schizophrenia, non-schizophrenia suicide and control groups was compared by one-way ANOVA analysis, followed by Bonferroni´s post-hoc analysis.

Pearson´s correlation r coefficient was calculated to test the possible associations between independent covariables, (age, PMD and storage time) and GNAI1 and GNAQ protein expression in schizophrenia, non-schizophrenia suicide and control groups. When correlation was significant, analysis of covariance (ANCOVA) was performed between groups controlling for the independent covariable. ANCOVA analyses were carried out by using InVivoStat statistical software.

Three group comparisons (control, schizophrenia and non-schizophrenia suicide) were made by one-way ANOVA, followed by Bonferroni´s post-hoc analysis. Differences were considered significant at $p<0.05$.

# Results

## 4.1 Functional selectivity of different serotonin 5-$HT_{2A}$R antagonists in post-mortem human brain

### 4.1.1 Concentration-effect of different drugs on $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein coupling to 5-$HT_{2A}$R in post-mortem human PFC

Concentration-response curves in presence of increasing concentration of different drugs were used for the functional coupling characterization of 5-$HT_{2A}$R to different Gα-proteins. Under this experimental condition, responses of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins **(Figure 4.1.A)** to increasing concentrations of MDL-11,939 ($10^{-10}$–$10^{-4}$ M) or ketanserin ($10^{-10}$–$10^{-4}$ M) were unaltered, suggesting that these drugs act as neutral antagonists for the $G\alpha_{i1}$-protein-mediated pathway. In contrast, other drugs such as altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin and eplivanserin displayed a concentration-dependent inhibitory response, which points out that these drugs were able to decrease basal constitutive activity.

When the canonical $G\alpha_{q/11}$-protein pathway of 5-$HT_{2A}$R was studied **(Figure 4.1.B)**, ketanserin increased [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-protein, displaying agonist properties. Conversely, altanserin, pimavanserin, nelotanserin and MDL-11,939 presented null effect on $G\alpha_{q/11}$-protein pathway. Finally, ritanserin, volinanserin and eplivanserin displayed concentration-response inhibitions of [$^{35}$S]GTPγS binding to the canonical pathway.

Therefore, it might be concluded that only MDL-11,939 acts as neutral antagonist for $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein-coupling of 5-$HT_{2A}$R in human brain cortex. In contrast, the rest of drugs displayed functional selectivity between 5-$HT_{2A}$R coupling to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins. The corresponding $E_{max}$/$I_{max}$ and $EC_{50}$/$IC_{50}$ values of concentration-response curves for each drug are shown in **Table 4.1.**

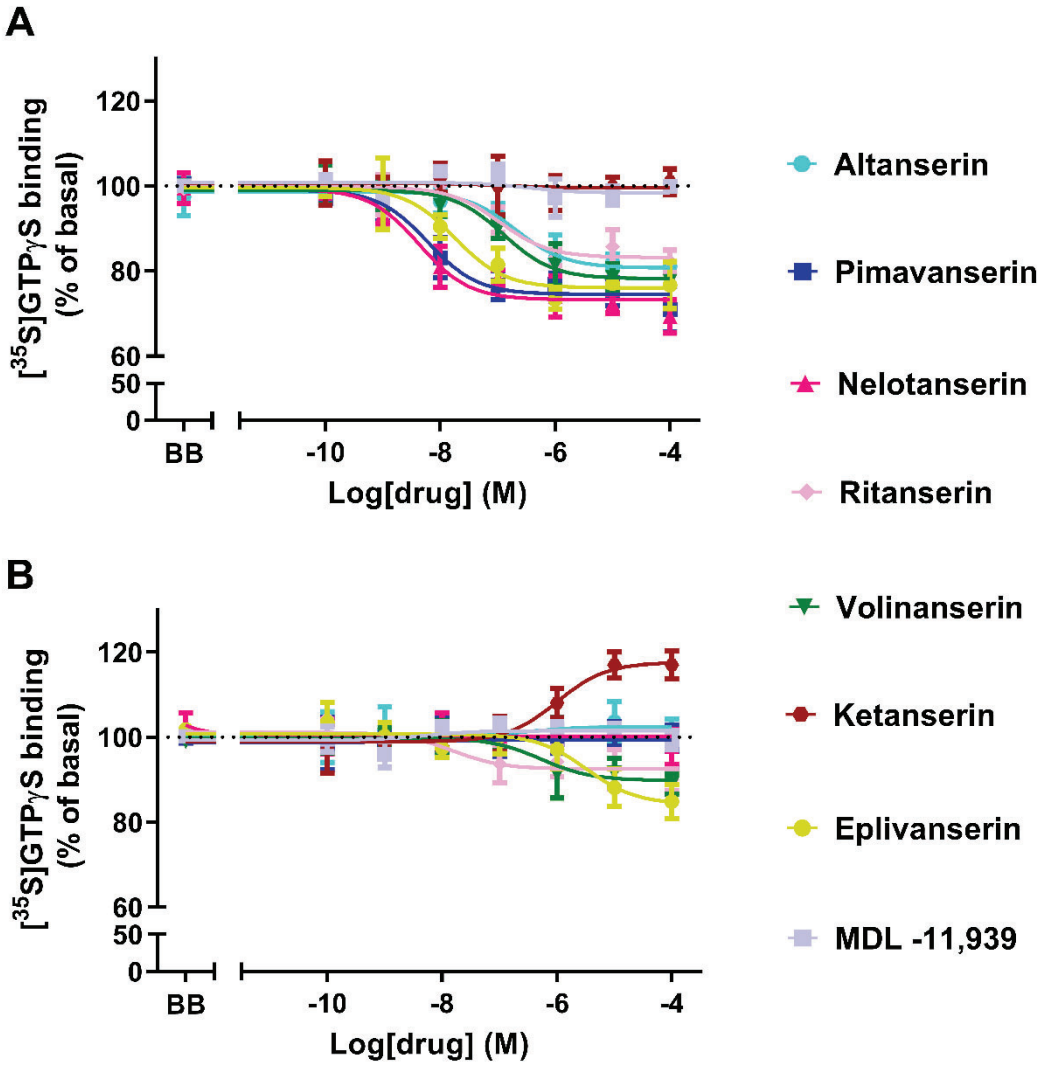



**Figure 4.1:** Concentration-response curves of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in response to addition of different 5-$HT_{2A}$R drugs. Concentration-response stimulation or inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- (Figure 4.1.A) and $G\alpha_{q/11}$- (Figure 4.1.B) proteins by altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939 was performed in human PFC membranes. The 100% dashed line denotes the specific basal [$^{35}$S]GTPγS binding to each Gα-protein (BB). Each point represents the mean±SEM value from independent experiments carried out in duplicate and triplicate.

The accurate determination of efficacy is essential for ligand bias detection. Analysis of concentration-response curves measures drug efficacy and potency, which are represented by maximal inhibitory or stimulatory responses ($E_{max}$/$I_{max}$), and the concentration that promotes half-maximal effect ($EC_{50}$/$IC_{50}$), respectively. Pimavanserin and nelotanserin showed the highest efficacy to decrease in almost a 30% [$^{35}$S]GTPγS basal binding to $G\alpha_{i1}$-protein ($I_{max}$=-27±2% and -25±2%) as shown in **Table 4.1**. In the same way, nelotanserin and pimavanserin also showed highest potency ($IC_{50}$=8.4±0.3 nM and 8.2±0.3 nM) (**Table 4.1**). None of the drugs showed intrinsic positive efficacy on the [$^{35}$S]GTPγS basal binding to $G\alpha_{i1}$-protein. However, ketanserin was able to increase the [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-protein by a 18%, with a potency of 994 nM (**Table 4.1**). Eplivanserin decreased the [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-protein by a 16%, which corresponded to the highest inhibitory efficacy. In contrast, eplivanserin had the lowest potency ($IC_{50}$=3268 nM). In terms of potency, ritanserin showed the lowest concentration required to reach the half-maximal effect of [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins ($IC_{50}$= 15.2 nM) (**Table 4.1**).

**Table 4.1:** Pharmacological parameters of the concentration-response curves of the specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in response to addition of different 5-$HT_{2A}$R drugs in human PFC membranes. Data are shown as mean±SEM of n independent experiments carried out in duplicate or triplicate. $E_{max}$ and $I_{max}$ represent, in % of basal values, the maximal stimulation and inhibition, respectively, estimated from non-linear fitting of the concentration-response curves. (-)log$EC_{50}$/$IC_{50}$ indicate the log of concentration (in nM) that promotes half-maximal stimulatory or inhibitory effect, and $IC_{50}$/$EC_{50}$ are the respective antilog values.

| | **$G\alpha_{i1}$-protein** | | | | **$G\alpha_{q/11}$-protein** | | | |
|---|---|---|---|---|---|---|---|---|
| | $E_{max}$/ $I_{max}$ (%) | (-)log$EC_{50}$ /$IC5_0$ | $IC_{50}$/$EC_{50}$ (nM) | n | $E_{max}$/ $I_{max}$ (%) | (-)log$EC_{50}$ /$IC_{50}$ | $IC_{50}$/$EC_{50}$ (nM) | n |
| Altanserin | -19±3 | 6.7±0.4 | 205.3 | 6 | / | / | / | 6 |
| Pimavanserin | -25±2 | 8.2±0.3 | 6.1 | 5 | / | / | / | 8 |
| Nelotanserin | -27±2 | 8.4±0.3 | 4.3 | 5 | / | / | / | 6 |
| Ritanserin | -17±2 | 7.0±0.3 | 108.1 | 5 | -7±2 | 7.8±0.7 | 15.2 | 5 |
| Volinanserin | -22±2 | 6.9±0.2 | 125.5 | 6 | -10±2 | 6.3±0.7 | 530.9 | 6 |
| Ketanserin | / | / | / | 5 | 18±2 | 6.0±0.3 | 994.1 | 7 |
| Eplivanserin | -24±2 | 7.8±0.2 | 17.8 | 7 | -16±4 | 5.5±0.5 | 3268 | 6 |
| MDL-11,939 | / | / | / | 5 | / | / | / | 5 |

### 4.1.2 Evaluation of maximal effect of different drugs on $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{i3}$-, $G\alpha_{o}$-, and $G\alpha_{q/11}$-protein coupling to 5-$HT_{2A}$R in post-mortem human PFC

In order to elucidate the effects of previously evaluated drugs on [$^{35}$S]GTPγS binding, not only to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins but also to other PTX-sensitive Gα-proteins (inhibitory $G\alpha_{i/o}$-proteins), a new set of experiments was performed. For these experiments, [$^{35}$S]GTPγS scintillation proximity assays (SPA) coupled to immunoprecipitation with different selective antibodies against $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{i3}$-, $G\alpha_{o}$- and $G\alpha_{q/11}$-protein subtypes were performed at one single concentration (10 μM). This concentration induces effects very close to maximal efficacy. Altanserin, pimavanserin and eplivanserin induced a statistically relevant inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins, while they did not show any effect on other inhibitory Gα-proteins (**Table 4.2**). Under these experimental conditions, nelotanserin and ritanserin decreased [$^{35}$S]GTPγS basal binding to $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{i3}$-, and $G\alpha_{o}$-proteins (**Table 4.2**). Volinanserin promoted inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{o}$-proteins (**Table 4.2**). Ketanserin and MDL-11,939 were unable to modify [$^{35}$S]GTPγS binding to inhibitory $G\alpha_{i/o}$ proteins (**Table 4.2**). In general, the maximal inhibitory effects were observed in $G\alpha_{i1}$-proteins (**Table 4.2**).

The responses of [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins, after addition of different drugs, were similar to those previously observed (see 4.1.1). Thus ritanserin, volinanserin and eplivanserin induced an inhibitory effect on [$^{35}$S]GTPγS binding, whereas ketanserin promoted stimulation (**Table 4.2**).

**Table 4.2**: Stimulatory or inhibitory effect induced by one single concentration (10 μM) of different drugs (altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939) on the specific [$^{35}$S]GTPγS binding to inhibitory $G\alpha_{i/o}$- and $G\alpha_{q/11}$-proteins in human PFC membranes. Data are mean±SEM of n independent experiments carried out in duplicate or triplicate, and express the percentage values related to basal [35S]GTPγS binding (0%). Data were analyzed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001).

| Drug | $G\alpha_{i1}$ | $G\alpha_{i2}$ | $G\alpha_{i3}$ | $G\alpha_{o}$ | $G\alpha_{q/11}$ |
|---|---|---|---|---|---|
| Altanserin | -12±2*** (n=12) | -1±3 (n=10) | 2±1 (n=11) | -4±5 (n=6) | 4±2 (n=12) |
| Pimavanserin | -20±3*** (n=10) | -2±2 (n=10) | 3±2 (n=11) | -1±3 (n=5) | 3±2 (n=13) |
| Nelotanserin | -22±3*** (n=9) | -17±4** (n=10) | -11±4* (n=8) | -6±2* (n=5) | 3±3 (n=11) |
| Ritanserin | -18±2*** (n=9) | -15±4* (n=6) | -10±3* (n=18) | -15±5* (n=7) | -11±3** (n=11) |
| Volinanserin | -16±2*** (n=9) | -2±3 (n=9) | -3±3 (n=9) | -4±1* (n=7) | -7±3* (n=10) |
| Ketanserin | -1±1 (n=5) | 2±4 (n=6) | -2±3 (n=7) | -1±2 (n=5) | 20±3** (n=7) |
| Eplivanserin | -19±5* (n=5) | -2±4 (n=6) | 0±5 (n=7) | 5±2 (n=5) | -17±3*** (n=9) |
| MDL-11,939 | 3±3 (n=7) | 0±2 (n=5) | -1±4 (n=5) | 0±1 (n=5) | 0±2 (n=7) |

### 4.1.3 Involvement of 5-$HT_{2A}$R in the effect induced by different drugs on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in post-mortem human PFC

The described findings in previous paragraphs suggest the involvement of 5-$HT_{2A}$R in the modulation of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins because evaluated drugs share activity on 5-HT2AR. In order to confirm the involvement of 5-$HT_{2A}$R, antagonism assays with selective 5-$HT_{2A}$R drugs were performed. For this purpose, altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939 were added to SPA assays at 10 μM concentration in presence of ketanserin (10 μM), volinanserin (1 μM) or MDL-11,939 (1 μM). Ketanserin ($pK_i$=8.5), volinanserin ($pK_i$=8.73) and MDL-11,939 ($pK_i$=7.58) were chosen as selective antagonist against $5HT_{2A}$R vs $5HT_{2C}$R, according to previous reports (Knight et al., 2004; Bonhaus et al., 1995).

The selectivity of different drugs for 5-$HT_{2A}$R was analysed by repeated measures one-way ANOVA, where the drug alone was compared with the drug co-incubated with the antagonist. A further Bonferroni´s post-hoc test would delineate the specific inhibition of different antagonists.

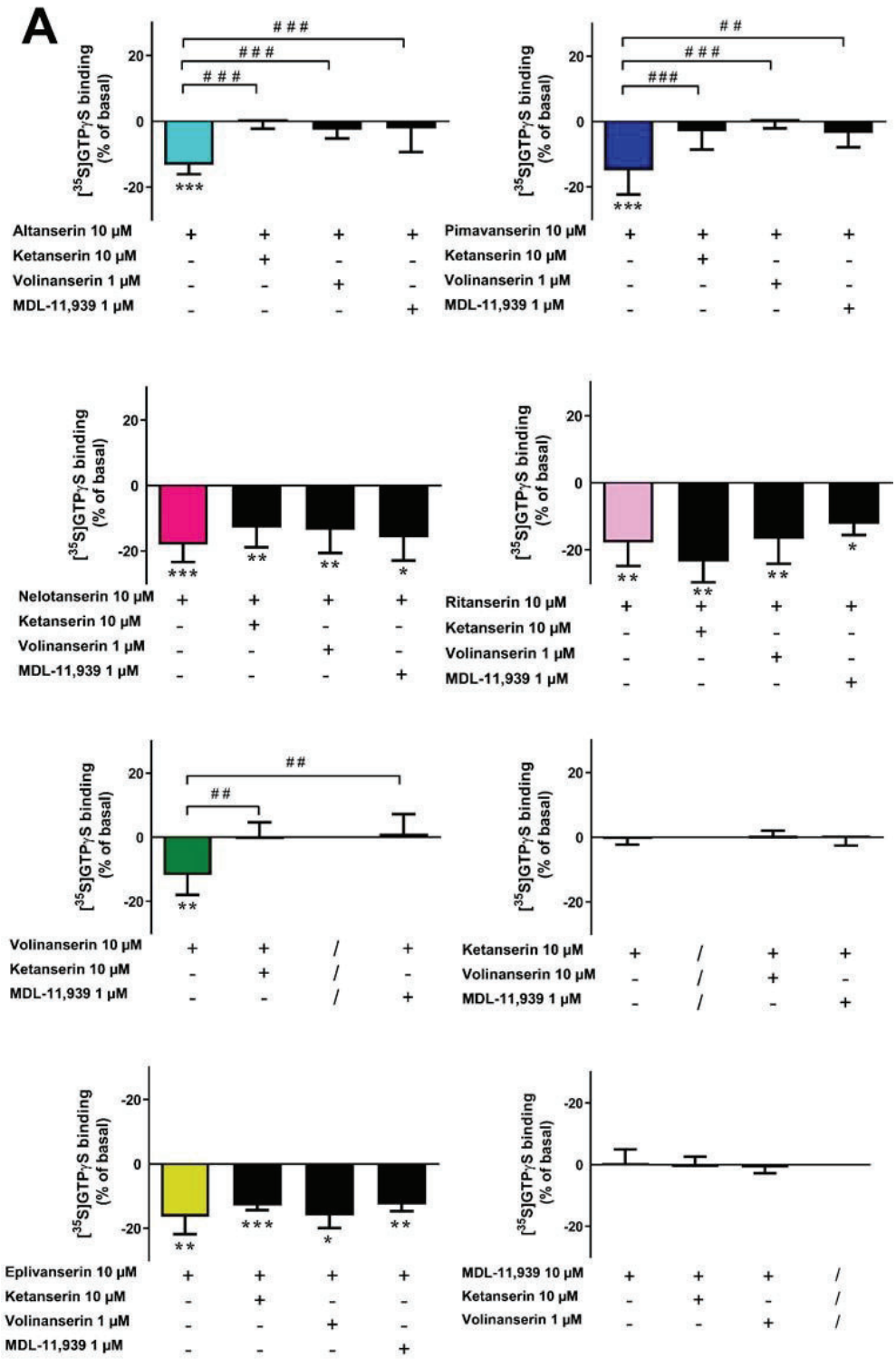
Ketanserin, volinanserin and MDL-11,939 blocked the inhibitory effects exerted by altanserin (-13±1%) ($F[3,18]=13.28$, $p<0.0001$) and pimavanserin (-15±3%) ($F[3,19]=11.80$, $p=0.0001$), on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (**Tables 4.3 and 4.4**) (**Figure 4.2.A**). Moreover, since volinanserin had unexpectedly demonstrated inhibitory effect on this activity (-12±3%), ketanserin and MDL-11,939 were also tested as potential antagonists on this effect. A full blockage of volinanserin-induced inhibition was observed ($F[2,15]=9.81$, $p=0.0019$) (**Tables 4.3 and 4.4**) (**Figure 4.2.A**), suggesting partial inverse agonism of this compound on the [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins.

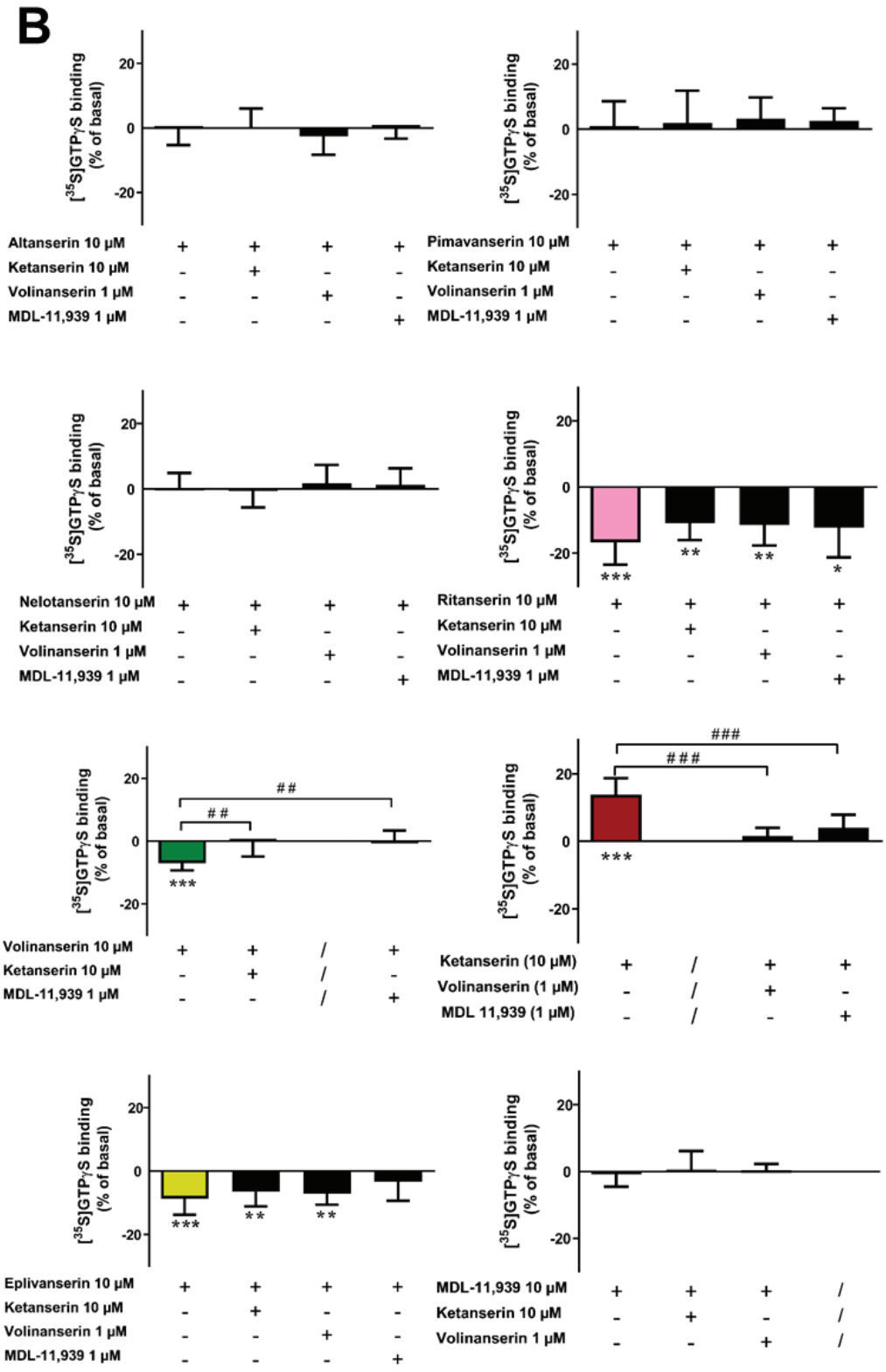
In contrast, ketanserin, volinanserin and MDL-11,939 did not exert any antagonism on inhibitory effects of nelotanserin (-19±2%) ($F[3,19]=0.71$, $p=0.5586$), ritanserin (-18±3%) ($F[3,16]=2.77$, $p=0.3422$) and eplivanserin (-16±2%) ($F[3,16]=1.58$, $p=0.2287$) on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (**Tables 4.3 and 4.4**) (**Figure 4.2.A**). In conclusion, the inhibitory effect induced by these three drugs should be mediated via another GPCR.

On the other hand, ketanserin, volinanserin and MDL-11,939 were not able to antagonize the inhibition induced by ritanserin (-17±2%) ($F[3,23]=1.18$, $p=0.1337$) and eplivanserin (-9±1%) ($F[3,30]=1.81$, $p=0.1531$) on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins (**Tables 4.3 and 4.4**) (**Figure 4.2.B**). This finding suggests that ritanserin- and eplivanserin-induced inhibitions were not mediated by 5-$HT_{2A}$R. In contrast, the small inhibition induced by volinanserin (-7±1%) was sensitive to ketanserin and MDL-11,939 ($F[2,16]=9.9$, $p<0.0016$) (**Tables 4.3 and 4.4**) (**Figure 4.2.B**). Moreover, the stimulation exerted by ketanserin (14±2%) on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was blocked in presence of volinanserin and MDL-11,939 ($F[2,24]=21.53$, $p<0.0001$), concluding that the agonist effect of ketanserin was mediated via 5-$HT_{2A}$R (**Tables 4.3 and 4.4**) (**Figure 4.2.B**).

**Table 4.3**: Summary of one-way ANOVA analysis of variance performed to test the selectivity of the drugs studied for $5HT_{2A}R$. In order to determine this selectivity, all drugs were co-incubated with putative antagonists ketanserin (10 μM), volinanserin (1 μM) or MDL-11,939 (1 μM). Significant p values are shown in **bold.**

| Gα-protein | **$G\alpha_{i1}$-protein** | | | | **$G\alpha_{q/11}$-protein** | | | |
|---|---|---|---|---|---|---|---|---|
| Drug (10 μM) | DFn | DFd | F | p | DFn | DFd | F | p |
| Altanserin | 3 | 18 | 13.28 | **<0.0001** | 3 | 18 | 0.30 | 0.8217 |
| Pimavanserin | 3 | 19 | 11.80 | **0.0001** | 3 | 25 | 0.44 | 0.7260 |
| Nelotanserin | 3 | 16 | 1.58 | 0.2338 | 3 | 26 | 0.31 | 0.8180 |
| Ritanserin | 3 | 16 | 2.78 | 0.0752 | 3 | 23 | 1.18 | 0.3380 |
| Volinanserin | 2 | 15 | 9.81 | **0.0019** | 2 | 16 | 9.90 | **0.0016** |
| Ketanserin | 2 | 16 | 0.19 | 0.8270 | 2 | 24 | 21.53 | **<0.0001** |
| Eplivanserin | 3 | 16 | 1.581 | 0.2330 | 3 | 30 | 1.81 | 0.1670 |
| MDL-11,939 | 2 | 14 | 0.20 | 0.8184 | 2 | 15 | 0.21 | 0.8164 |


A
[35S]GTPγS binding (% of basal)
Altanserin 10 µM
Ketanserin 10 µM
Volinanserin 1 µM
MDL-11,939 1 µM
Pimavanserin 10 µM
Nelotanserin 10 µM
Ritanserin 10 µM
Volinanserin 10 µM
Ketanserin 10 µM
MDL-11,939 1 µM
Eplivanserin 10 µM
MDL-11,939 10 µM


B
[35S]GTPγS binding (% of basal)
20
0
-20
Altanserin 10 µM + + + +
Ketanserin 10 µM - + - -
Volinanserin 1 µM - - + -
MDL-11,939 1 µM - - - +
Pimavanserin 10 µM + + + +
Ketanserin 10 µM - + - -
Volinanserin 1 µM - - + -
MDL-11,939 1 µM - - - +
Nelotanserin 10 µM + + + +
Ketanserin 10 µM - + - -
Volinanserin 1 µM - - + -
MDL-11,939 1 µM - - - +
Ritanserin 10 µM + + + +
Ketanserin 10 µM - + - -
Volinanserin 1 µM - - + -
MDL-11,939 1 µM - - - +
***
**
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*
##
##
***
Volinanserin 10 µM + + / +
Ketanserin 10 µM - + / -
MDL-11,939 1 µM - - / +
###
###
***
Ketanserin (10 µM) + / + +
Volinanserin (1 µM) - / + -
MDL 11,939 (1 µM) - / - +
***
**
**
Eplivanserin 10 µM + + + +
Ketanserin 10 µM - + - -
Volinanserin 1 µM - - + -
MDL-11,939 1 µM - - - +
MDL-11,939 10 µM + + + /
Ketanserin 10 µM - + - /
Volinanserin 1 µM - - + /

**Figure 4.2**: Antagonism of the stimulatory or inhibitory effect induced by one single concentration (10 μM) of different drugs (altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939) on the specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- (**Figure 4.2.A**) and $G\alpha_{q/11}$-proteins (**Figure 4.2.B**) in human PFC membranes. These different drugs were co-incubated with the selective 5-$HT_{2A}$R antagonists ketanserin (10 μM), volinanserin (1 μM) or MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins are expressed as 0%, and stimulatory or inhibitory effects on respective basal are shown as percentage of basal values. Bars represent mean±SEM percentage values of independent experiments carried out in duplicate or triplicate. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001) and one-way ANOVA followed by Bonferroni´s post-hoc test antagonism effects ($^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001).

**Table 4.4**: Numerical values of results expressed in **Figure 4.2.** Antagonism of stimulatory or inhibitory effect induced by one single concentration (10 μM) of different drugs (altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939) on the specific [$^{35}$S]GTPγS binding to inhibitory $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in human PFC membranes. These different drugs were co-incubated with the selective 5-$HT_{2A}$R antagonists ketanserin (10 μM), volinanserin (1 μM) or MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins are expressed as 0%, and stimulatory or inhibitory effects on respective basal are shown as percentage of basal values. Values represent mean±SEM percentage values of n independent experiments carried out in duplicate or triplicate. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001) and one-way ANOVA followed by Bonferroni´s post-hoc test for antagonism effects ($^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001).

**$G\alpha_{i1}$-protein**

| | Drug alone 10 µM | + ketanserin 10 µM | + volinanserin 1 µM | + MDL-11,939 1 µM |
|---|---|---|---|---|
| Altanserin | -13±1*** (n=7) | 0±1### (n=5) | -3±1### (n=5) | -2±3### (n=5) |
| Pimavanserin | -15±3*** (n=8) | -1±2### (n=6) | 0±1### (n=5) | -4±2## (n=5) |
| Nelotanserin | -19±2*** (n=8) | -13±3** (n=5) | -14±3** (n=5) | -16±3* (n=5) |
| Ritanserin | -18±3** (n=5) | -23±3** (n=5) | -17±3** (n=5) | -12±1** (n=5) |
| Volinanserin | -12±3** (n=6) | 0±2## (n=6) | / | 1±3## (n=6) |
| Ketanserin | -1±1 (n=5) | / | -2±3 (n=7) | 0±1 (n=7) |
| Eplivanserin | -16±2** (n=5) | -13±1*** (n=5) | -16±2*** (n=5) | -13±1*** (n=5) |
| MDL-11,939 | 0±2 (n=6) | 0±1 (n=5) | -1±2 (n=6) | / |

**$G\alpha_{q/11}$-protein**

| | Drug alone 10 µM | + ketanserin 10 µM | + volinanserin 1 µM | + MDL-11,939 1 µM |
|---|---|---|---|---|
| Altanserin | 0±2 (n=7) | 0±3 (n=5) | -3±3 (n=5) | 0±1 (n=5) |
| Pimavanserin | 1±2 (n=10) | 2±4 (n=7) | 3±2 (n=7) | 3±1 (n=7) |
| Nelotanserin | 0±2 (n=9) | -1±2 (n=7) | 2±2 (n=7) | 1±2 (n=7) |
| Ritanserin | -17±2*** (n=10) | -11±2** (n=5) | -11±3** (n=6) | -12±4* (n=6) |
| Volinanserin | -7±1*** (n=7) | 0±2## (n=5) | / | 0±1## (n=7) |
| Ketanserin | 14±2*** (n=7) | / | -1±1### (n=10) | 2±2### (n=10) |
| Eplivanserin | -9±1*** (n=13) | -7±2** (n=7) | -7±1** (n=7) | -3±2 (n=7) |
| MDL-11,939 | -1±1 (n=6) | 1±2 (n=6) | 0±2 (n=6) | / |

### 4.1.4 Evaluation of the maximal effect of different drugs on $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein coupling to 5-$HT_{2A}R$ in knock-out 5-$HT_{2A}R^{(-/-)}$ and wild-type 5-$HT_{2A}R^{(+/+)}$ mice

To further confirm the role of 5-$HT_{2A}R$ in the stimulatory or inhibitory effect exerted on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins, SPA experiments were performed in brain tissue of knock-out 5-$HT_{2A}R^{(-/-)}$ and wild-type 5-$HT_{2A}R^{(+/+)}$ mice. Brain membranes were incubated with a single concentration (10 μM) of altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939 as well as specific antibodies against $G\alpha_{i1}$- (**Figure 4.3.A**) and $G\alpha_{q/11}$-proteins (**Figure 4.3.B**).

There were not differences in basal values on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins between 5-$HT_{2A}R^{(+/+)}$ and 5-$HT_{2A}R^{(-/-)}$ mice. Thus, the basal binding values for $G\alpha_{i1}$-proteins in wild-type mice were 3824±428 CCPM, and 3971±428 CCPM in knock-out mice ($p=0.8115$, $t=0.24$, $df=14$). Similarly, the basal binding values for $G\alpha_{q/11}$-protein were 3160±329 CCPM in wild-type mice, and 3224±224 CCPM in knock-out mice ($p=0.8779$, $t=0.16$, $df=16$).

The comparison between both genotypes in response to different drugs was performed by a repeated-measures two-way ANOVA, where conditions were drug and genotype. This analysis was performed twice, corresponding to evaluation of [$^{35}$S]GTPγS binding responses for each Gα-protein. Since the effects induced by drugs are variable, it was expected to find a significant interaction drug x genotype. A further Bonferroni's post-hoc test would delineate the specific drugs displaying differential effects between wild-type and knock-out animals.

In agreement with the results in human brain samples, when wild-type mice brain cortex membranes where incubated with altanserin (-10±3%), pimavanserin (-13±2%), nelotanserin (-12±2%), ritanserin (-12±3%), volinanserin (-11±2%) and eplivanserin (-12±3%), all of them exerted an

inhibitory effect on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (**Table 4.5**) (**Figure 4.3.A**). Furthermore, ketanserin and MDL-11,939 did not change basal [$^{35}$S]GTPγS binding (**Table 4.5**) (**Figure 4.3.A**).The significant inhibition exerted on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins by altanserin, pimavanserin and volinanserin was absent in knock-out mice membranes (**Table 4.5**) (**Figure 4.3.A**) These results confirm involvement of 5-$HT_{2A}$R in the inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins. On the other hand, the inhibition exerted by nelotanserin, ritanserin and eplivanserin was also observed in knock-out mice, i.e. absence of 5-$HT_{2A}$R (**Table 4.5**) (**Figure 4.3.A**). This results further support that inhibitory effects on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins exerted by these three drugs are not mediated by 5-$HT_{2A}$R.

Next, [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was studied in wild-type 5-$HT_{2A}R^{(+/+)}$ mice. In a similar way to results in human frontal cortex, ritanserin (-8±1%), volinanserin (-14±2%) and eplivanserin (-10±1%) decreased [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins (**Table 4.5**) (**Figure 4.3.B**). Furthermore, altanserin, pimavanserin, nelotanserin and MDL-11,939 did not modify the basal [$^{35}$S]GTPγS binding of this canonical pathway (**Table 4.5**) (**Figure 4.3.B**). Moreover, ketanserin induced a stimulatory (18±4%) response in wild-type animals, as described in human brain (**Table 4.5**) (**Figure 4.3.B**). When these drugs were studied in knock-out mice, their effect was null for volinanserin and ketanserin, indicating that their effects were mediated by 5-$HT_{2A}$R. In contrast, ritanserin and eplivanserin decreased [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in knock-out mice as in wild-type mice, suggesting the lack of involvement of 5-$HT_{2A}$R on these effects (**Table 4.5**) (**Figure 4.3.B**).

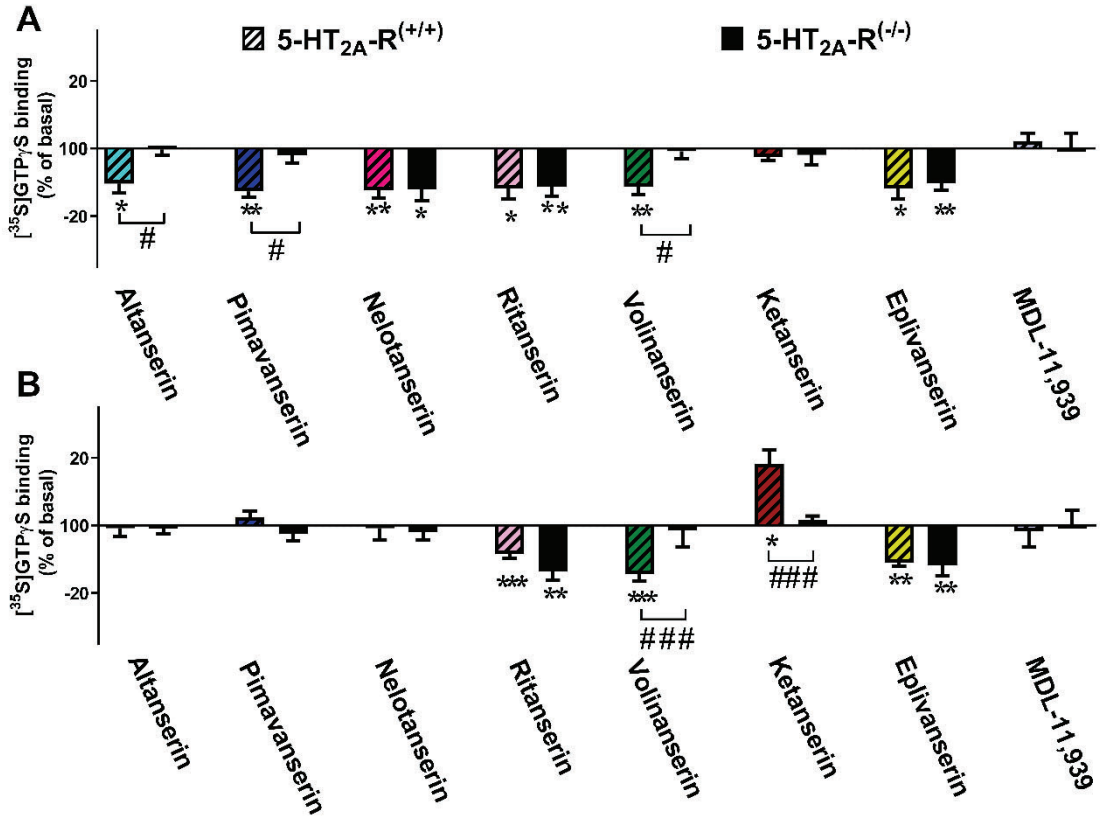



**Figure 4.3**: Stimulatory or inhibitory effect induced by one single concentration (10 μM) of different drugs (altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939) on the specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- (**Figure 4.3.**A) and $G\alpha_{q/11}$- (**Figure 4.3.B**) proteins in brain cortex membranes of wild-type 5-$HT_{2A}R^{(+/+)}$ and knock-out 5-$HT_{2A}R^{(-/-)}$ mice. Bars are mean±SEM of independent experiments carried out in duplicate or triplicate, and express the percentage values related to basal [$^{35}$S]GTPγS binding (0%). Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001) and two-way ANOVA followed by Bonferroni´s post-hoc test for drug x genotype effects ($^{\#}$p<0.05, $^{\#\#\#}$p<0.001).

**Table 4.5**: Numerical values of results expressed in **Figure 4.3**. Stimulatory or inhibitory effect induced by one single concentration (10 μM) of different drugs (altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939) on the specific [$^{35}$S]GTPγS binding to $G\alpha_{i/o}$- and $G\alpha_{q/11}$-proteins in brain cortex membranes of wild-type 5-$HT_{2A}R^{(+/+)}$ and knock-out 5-$HT_{2A}R^{(-/-)}$ mice. Values are mean±SEM of n independent experiments carried out in duplicate or triplicate and express the percentage values related to basal [$^{35}$S]GTPγS binding (0%). Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001).

| | **$G\alpha_{i1}$-protein** | | **$G\alpha_{q/11}$-protein** | |
|---|---|---|---|---|
| | 5-$HT_{2A}R^{(+/+)}$ | 5-$HT_{2A}R^{(-/-)}$ | 5-$HT_{2A}R^{(+/+)}$ | 5-$HT_{2A}R^{(-/-)}$ |
| Altanserin | -10±3* (n=5) | 0±2 (n=6) | -1±3 (n=6) | -1±2 (n=7) |
| Pimavanserin | -13±2** (n=5) | -2±2 (n=6) | 2±2 (n=6) | -4±2 (n=7) |
| Nelotanserin | -12±2** (n=5) | -12±3* (n=6) | -1±3 (n=5) | -2±2 (n=8) |
| Ritanserin | -12±3* (n=5) | -11±3** (n=6) | -8±1*** (n=8) | -14±3** (n=7) |
| Volinanserin | -11±2** (n=5) | -1±2 (n=5) | -14±2*** (n=8) | -1±5 (n=8) |
| Ketanserin | -2±1 (n=5) | -2±3 (n=5) | 18±4* (n=5) | 2±1 (n=7) |
| Eplivanserin | -11±3* (n=5) | -10±2** (n=6) | -10±2** (n=4) | -12±3** (n=5) |
| MDL-11,939 | 2±2 (n=5) | 0±4 (n=6) | -2±5 (n=6) | 0±4 (n=6) |

**Table 4.6**: Summary of the repeated measures two-way ANOVA analysis of the effects of different drugs on wild-type 5-$HT_{2A}R^{(+/+)}$ and knock-out 5-$HT_{2A}R^{(-/-)}$ mice. The results of a drug x genotype interaction suggest that the response to the drugs is variable between both mice genotypes. A further post-hoc analysis was performed to discriminate differences between wild-type and knock-out animals for each drug. Results are shown in **Figures 4.3.A** and **4.3.B.** Significant p values are shown in **bold.**

| | **$G\alpha_{i1}$-protein** | | | | **$G\alpha_{q/11}$-protein** | | | |
|---|---|---|---|---|---|---|---|---|
| | DFn | DFd | F | P | DFn | DFd | F | p |
| Interaction | 7 | 70 | 2.84 | **0.0116** | 7 | 105 | 7.02 | **<0.0001** |
| Drug | 7 | 70 | 8.25 | **<0.0001** | 7 | 105 | 20.24 | **<0.0001** |
| Genotype | 1 | 70 | 10.44 | **0.0090** | 1 | 105 | 2.23 | 0.1558 |

### 4.1.5 Summary

5-$HT_{2A}$R expressed in human PFC displays functional coupling to the canonical Gαq/11-proteins but also to other cell pathways through inhibitory Gαi/o-proteins. Among them, $G\alpha_{i1}$-protein seems to be the preferential subtype involved in this alternative signaling pathway.

The functional coupling of 5-$HT_{2A}$R in human PFC shows constitutive activity because [$^{35}$S]GTPγS binding to different Gα-protein subunits can be inhibited by different 5-$HT_{2A}$R drugs, previously assumed to be neutral antagonist. Therefore, these compounds should be considered potential inverse agonists. The blockage of inverse agonism by selective 5-$HT_{2A}$R neutral antagonists, and the absence of this pharmacological feature in 5-$HT_{2A}R^{(-/-)}$ mice allow us to identify them as selective 5-$HT_{2A}$R inverse agonists.

Functional activity of the different drugs as inverse agonists of 5-$HT_{2A}$R may be different between $G\alpha_{q/11}$- and $G\alpha_{i1}$-protein pathways. Therefore, a biased inverse agonism is feasible for this receptor in human brain.

Among the different compounds tested, pimavanserin displays higher and more selective inverse efficacy in 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins, lacking of activity on $G\alpha_{q/11}$-protein activation. On the other hand, volinanserin could be considered a selective 5-$HT_{2A}$R inverse agonist with inverse efficacy on the coupling to both $G\alpha_{q/11}$- and $G\alpha_{i1}$-proteins. Therefore, these two drugs were selected as suitable tools for further studies of 5-$HT_{2A}$R constitutive activity in pathological conditions.

## 4.2 Evaluation of the functional coupling of 5-$HT_{2A}$R to Gα-protein subtypes in PFC of schizophrenia subjects, non-schizophrenia suicide subjects and matched controls by antibody-capture [$^{35}$S]GTPγS scintillation proximity assay (SPA)

### 4.2.1 Basal [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$- proteins in post-mortem PFC of schizophrenia subjects, non-schizophrenia suicide subjects and matched controls

Basal [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in cortical membranes was not statistically different between schizophrenia (162±7 fmol/mg prot), non-schizophrenia suicide (175±9 fmol/mg prot) and control (159±5 fmol/mg prot) groups (F[2,55]=1.13, p=0.35) (**Figure 4.4.A**). Similarly, basal [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was not statistically different between schizophrenia (191±9 fmol/mg prot), non-schizophrenia suicide (198±9 fmol/mg prot) and control subjects (210±11 fmol/mg prot) (F[2,56]=0.95, p=0.39) (**Figure 4.4.B**).

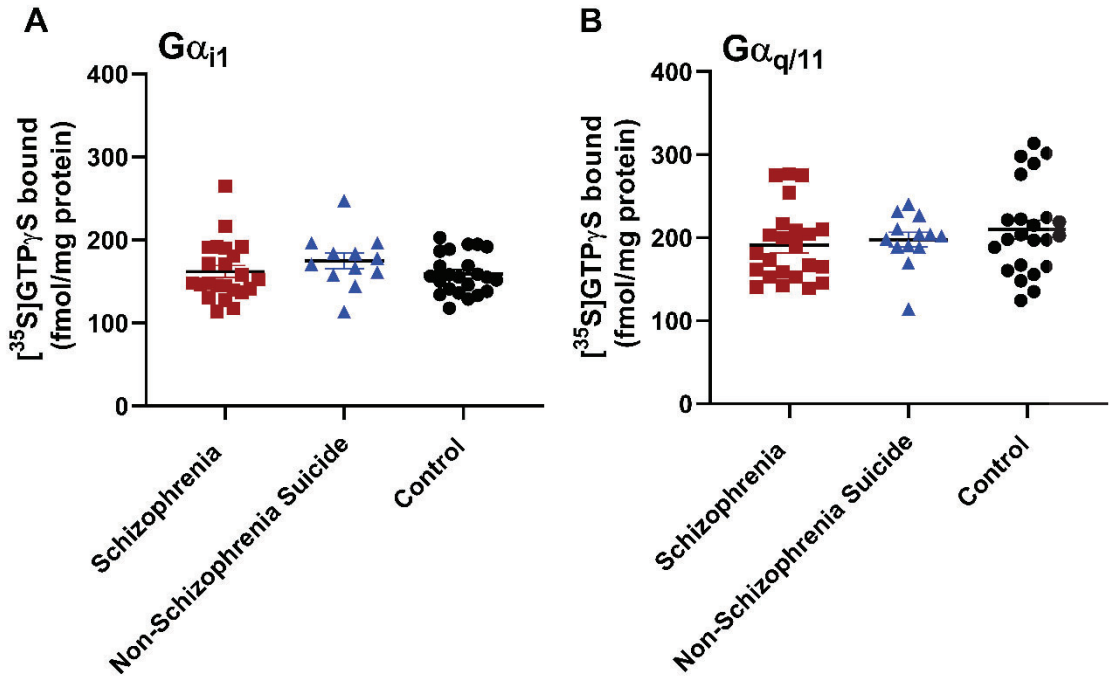



**Figure 4.4**: Individual values of basal [$^{35}$S]GTPγS binding to Gα$_{i1}$- (**A**) and Gα$_{q/11}$- (**B**) proteins in PFC membranes of schizophrenia (n=23) (red square), non-schizophrenia suicide (n=13) (blue triangle) and control subjects (n=23) (black circle). The lines represents mean±SEM values of each group.

In order to evaluate the influence of variables such as age at death, sex, PMD and storage time on basal [$^{35}$S]GTPγS binding to both Gα-proteins, linear correlation analyses were performed. Any of these potential variables showed correlation with [$^{35}$S]GTPγS basal binding values. The absence of relationship was also observed when considered separately those three population groups (**Table 4.7**).

**Table 4.7**: Effect of age (years), PMD (hours) and storage time (months) on basal [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins.

| **$G\alpha_{i1}$-protein basal binding (fmol/mg protein)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Schizophrenia** | | | **Non-schizophrenia suicide** | | | **Control** | | |
| | r | p | n | r | p | n | r | p | n |
| Age (years) | -0.2811 | 0.1939 | 23 | -0.1690 | 0.5995 | 13 | 0.0150 | 0.9458 | 23 |
| PMD (hours) | 0.1012 | 0.6459 | 23 | 0.2500 | 0.4332 | 13 | -0.0716 | 0.7452 | 23 |
| Storage time (months) | -0.0175 | 0.9368 | 23 | -0.0797 | 0.8054 | 13 | 0.0262 | 0.9056 | 23 |
| **$G\alpha_{q/11}$-protein basal binding (fmol/mg protein)** | | | | | | | | | |
| | **Schizophrenia** | | | **Non-schizophrenia suicide** | | | **Control** | | |
| | r | p | n | r | p | n | r | p | n |
| Age (years) | -0.2783 | 0.1986 | 23 | -0.3972 | 0.1790 | 13 | 0.1534 | 0.4847 | 23 |
| PMD (hours) | 0.1816 | 0.4070 | 23 | -0.1876 | 0.5403 | 13 | -0.0565 | 0.7978 | 23 |
| Storage time (months) | -0.0823 | 0.7071 | 23 | 0.1802 | 0.5558 | 13 | 0.0778 | 0.7245 | 23 |

### 4.2.2 Modulation of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins by the 5-$HT_{2A}$R inverse agonist pimavanserin in post-mortem PFC of schizophrenia subjects, non-schizophrenia suicide subjects and matched controls

Pimavanserin ($10^{-10}$-$10^{-6}$ M) displayed concentration-dependent inhibition curves in schizophrenia, non-schizophrenia suicide and control groups (**Figure 4.5**). No differences in potency of pimavanserin were observed between groups (schizophrenia: -log$IC_{50}$=7.94±0.1; non-schizophrenia suicide: -log$IC_{50}$=8.15±0.0; control: -logIC50=8.01±0.1) (F[2,56]=2.26, p=0.0871) (**Table 4.8**). The inhibitory effect of pimavanserin was higher in schizophrenia ($I_{max}$=-20±1%) than in control ($I_{max}$=-14±1%) and non-schizophrenia suicide ($I_{max}$=-14±1%) groups (F[2,56]=9.19, p=0.0004) (Table 4.8).

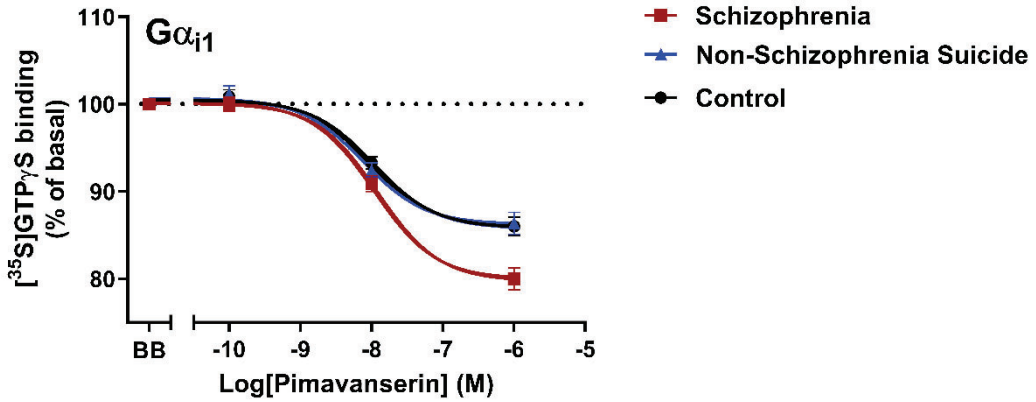



**Figure 4.5**: Concentration-response curves of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in response to pimavanserin in human PFC membranes. The 100% dashed line denotes specific basal [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (BB). Each point represents the mean±SEM value from independent experiments. See coanalysis results in the text.

**Table 4.8**: Pharmacological parameters of pimavanserin concentration-response curves of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and matched control subjects. $IC_{50}$ values were obtained as antilog of log$IC_{50}$. $I_{max}$ represents the experimental maximal inhibitory effect induced by pimavanserin, and is expressed as percentage of respective basal [$^{35}$S]GTPγS binding value**.** Values are mean±SEM of n independent subjects. Data were analysed using one-way ANOVA followed by Bonferroni's post-hoc test (**p<0.01 vs control and non-schizophrenia group).

| Group | Basal (fmol/ mg protein) | $IC_{50}$ (nM) | $I_{max}$ (%) | n |
|---|---|---|---|---|
| Schizophrenia | 162±7 | 11.9±2 | -20±1** | 23 |
| Non-schizophrenia suicide | 175±9 | 13.3±2 | -14±1 | 13 |
| Control | 159±5 | 7.7±1 | -14±1 | 23 |

An analysis of covariance (ANCOVA) was further performed to evaluate the potential influence of age at death, PMD and storage time. Sex differences were also evaluated. Any of these variables modified the results and, consistent with previous findings, differences between groups in $I_{max}$ values were maintained ($F[2,52]=6.84$, $p=0.0023$).

A complementary statistical comparison between groups was conducted by non-linear curve fitting coanalysis of all individual experiments. Coanalysis of concentration-response curves to pimavanserin demonstrated different patterns of schizophrenia respect to control ($F[3,177]=10.79$, $p<0.0001$), and non-schizophrenia suicide ($F[3,137]=8.16$, $p<0.0001$) groups (**Figure 4.5**). After these statistical differences between curves were obtained, further contrasts were performed to detect whether differences were attributable to changes in $I_{max}$ and/or $IC_{50}$ values between groups. Thus, curve fitting coanalysis confirmed that schizophrenia subjects showed a higher inhibitory effect of pimavanserin ($I_{max}$=-20%, 95%CI 22% to 18%) than control ($I_{max}$=-14%, 95%CI 16% to 13%) ($F[1,137]=22.29$, $p<0.0001$) and non-schizophrenia suicide subjects ($I_{max}$=-14%, 95%CI 16% to 12%) ($F[1,137]=22.29$, $p<0.0001$).

No differences were obtained for $IC_{50}$ values between schizophrenia ($IC_{50}$=12.14 nM, 95%CI 8.02 nM to 18.47 nM), non-schizophrenia suicide ($IC_{50}$=7.60 nM, 95%CI 3.74 nM to 13.84 nM) and control groups ($IC_{50}$=10.42 nM, 95%CI 6.21 nM to 17.19 nM).

Selectivity of the inhibitory effect exerted by pimavanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins was tested by using 5-$HT_{2A}$R neutral antagonist MDL-11,939 at 10 μM concentration. In schizophrenia subjects, pimavanserin maximal inhibitory effect on [$^{35}$S]GTPγS binding (-20±1%) was blocked by the antagonist ($p<0.0001$, $t=12.99$, $df=44$) (**Figure 4.6**). In non-schizophrenia suicide group, the maximal inhibition promoted by pimavanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (-14±1%) was also completely blocked by MDL-11,939 ($p<0.0001$, $t=6.82$, $df=24$) (**Figure 4.6**). As expected and previously observed (**See 4.1.3**) in control group, the maximal inhibition of [$^{35}$S]GTPγS binding induced by pimavanserin (-14±1%) was blocked by MDL-11,939 ($p<0.0001$, $t=8.02$, $df=43$) (**Figure 4.6**).

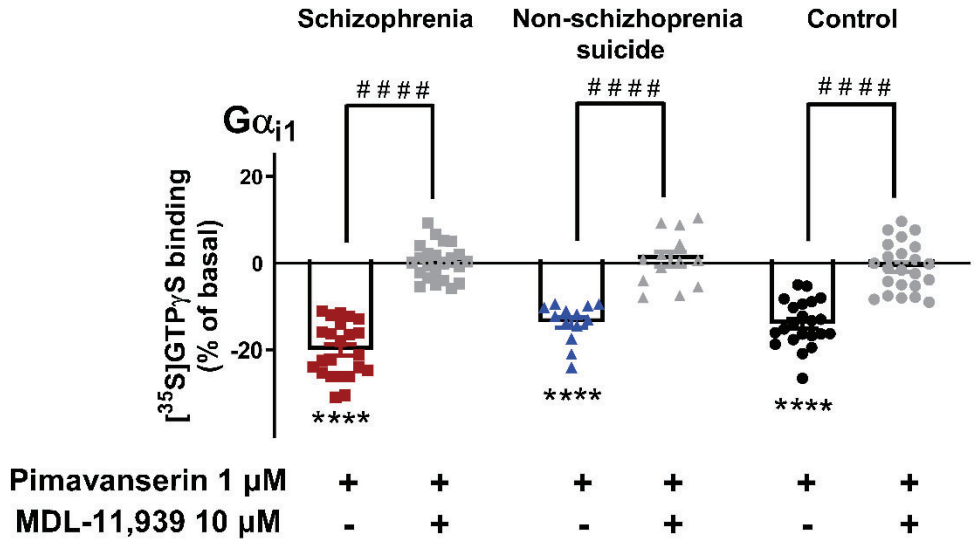



**Figure 4.6**: Antagonism of the inhibitory effect induced by a maximal concentration of pimavanserin (1 μM) on the specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and control subjects. Pimavanserin was co-incubated with the selective 5-$HT_{2A}$R antagonist MDL-11,939 (10 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins are expressed as 0%, and inhibitory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (****p<0.0001) and two sample t-test to compare pimavanserin effects in MDL-11,939 absence or presence (####p<0.0001).

Selective drug pimavanserin at one single concentration (1 μM) was also tested on the modulation of [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins by 5-$HT_{2A}$R in schizophrenia, non-schizophrenia suicide and control groups. As expected, and according to previous results (See 4.1.1), neither stimulatory nor inhibitory effects on basal [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-protein were observed (**Table 4.9**) (**Figure 4.7**). Consequently, no statistical differences were found between groups in the efficacy of pimavanserin ($F[2,56]=0.69$, $p=0.5081$).

**Table 4.9**: Pharmacological parameters of pimavanserin-induced effect at one single concentration (1 μM) on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and matched control subjects. $E_{max}/I_{max}$ represents experimental maximal stimulatory or inhibitory effect, respectively, induced by pimavanserin, and is expressed as percentage of respective basal [$^{35}$S]GTPγS binding value**.** Values are mean±SEM of n independent subjects. Data were analysed using one-way ANOVA.

| Group | Basal (fmol/ mg protein) | $IC_{50}$ (nM) | $I_{max}/E_{max}$ (%) | n |
|---|---|---|---|---|
| Schizophrenia | 191±9 | / | 2±1 | 23 |
| Non-schizophrenia suicide | 198±9 | / | 1±1** | 13 |
| Control | 210±11 | / | 0±1 | 23 |

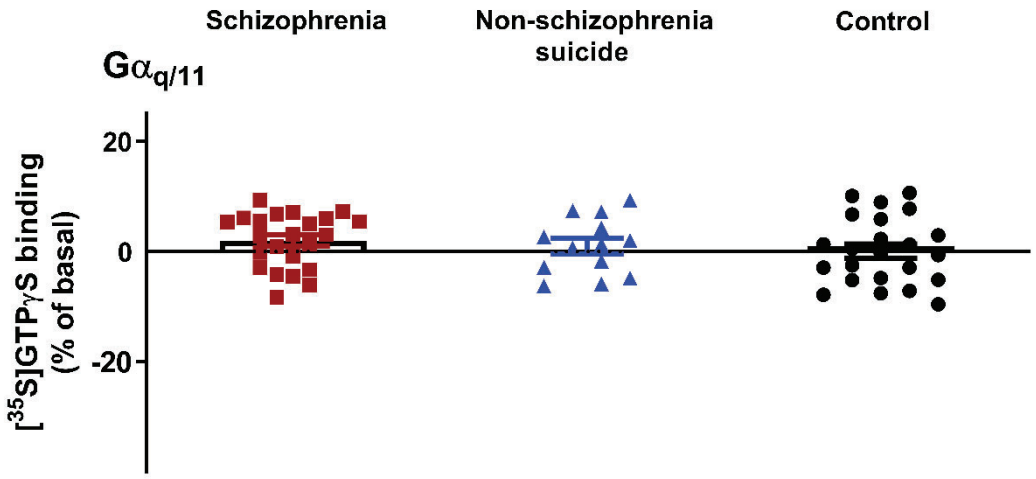



**Figure 4.7**: Pimavanserin-induced effect on the specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in human PFC membranes. Pimavanserin was added at 1 μM concentration. Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins are expressed as 0%, and inhibitory or stimulatory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments.

The modulation of [$^{35}$S]GTPγS binding to Gα$_{q/11}$-proteins by pimavanserin (1 μM) was also tested in presence of the 5-HT$_{2A}$R neutral antagonist MDL-1,939 (10 μM) in schizophrenia, non-schizophrenia suicide and control subjects (**Figure 4.8**). No changes on [$^{35}$S]GTPγS binding were observed in the pimavanserin null effect when antagonist was added (**Figure 4.8**).

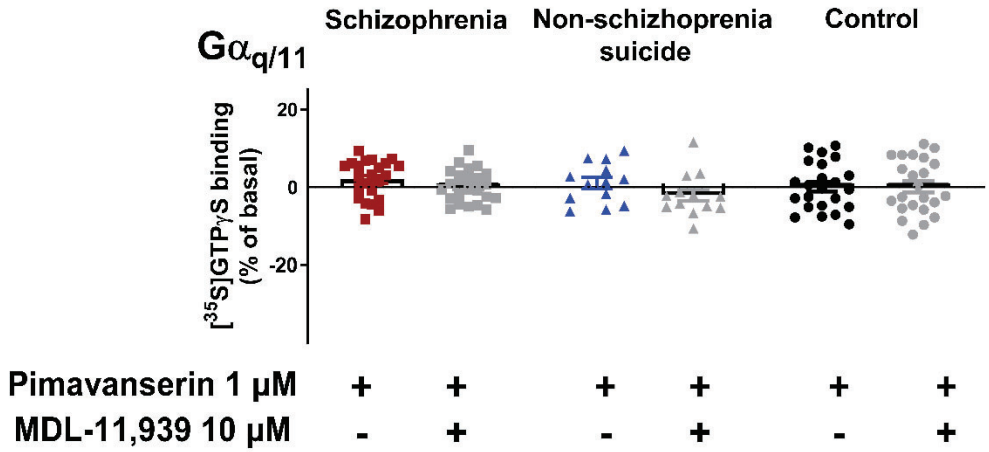



**Figure 4.8**: Antagonism of the effect induced by a single maximal concentration of pimavanserin (1 μM) on the specific [$^{35}$S]GTPγS binding to Gα$_{q/11}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and control subjects. Pimavanserin was co-incubated with the selective 5-HT$_{2A}$R antagonist MDL-11,939 (10 μM). Basal values of specific [$^{35}$S]GTPγS binding to Gα$_{q/11}$-proteins are expressed as 0%, and stimulatory or inhibitory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using two sample t-test to compare pimavanserin effects in absence or presence of MDL-11,939.

### 4.2.3 Modulation of the [$^{35}$S]GTPγS binding to Gα$_{i1}$- and Gα$_{q/11}$-proteins by the 5-HT$_{2A}$R inverse agonist volinanserin in post-mortem PFC of schizophrenia subjects, non-schizophrenia suicide subjects and matched controls

Given the absence of [$^{35}$S]GTPγS binding modulation by pimavanserin, the status of 5-HT$_{2A}$R coupling to Gα$_{q/11}$-proteins could not be quantified by using this drug. In order to evaluate whether the enhanced constitutive activity of 5-HT$_{2A}$R coupling to Gα$_{i1}$-proteins was selective for this pathway, or Gα$_{q/11}$-protein canonical pathway was also altered, another 5-HT$_{2A}$R inverse agonist should be tested. For this purpose, and according to previous findings, volinanserin was chosen as the most suitable pharmacological tool.

Volinanserin ($10^{-9}$-$10^{-5}$ M) displayed a concentration-dependent inhibition curves of [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins in schizophrenia, non-schizophrenia suicide and control groups (**Figure 4.9**). No differences in potency of volinanserin were observed between groups (schizophrenia: -logIC$_{50}$=6.45±0.1; non-schizophrenia suicide: -logIC$_{50}$=6.53±0.1; control: -logIC$_{50}$=6.54±0.1) (F[2,56]=0.5830, p=0.5616) (**Table 4.10**). The inhibitory effect of volinanserin was higher in schizophrenia subjects ($I_{max}$=-18±1%) than in control ($I_{max}$=-13±1%), as well as in non-schizophrenia suicide subjects ($I_{max}$=-14±1%) (F[2,56]=9.76, p=0.0002) (**Table 4.10**).

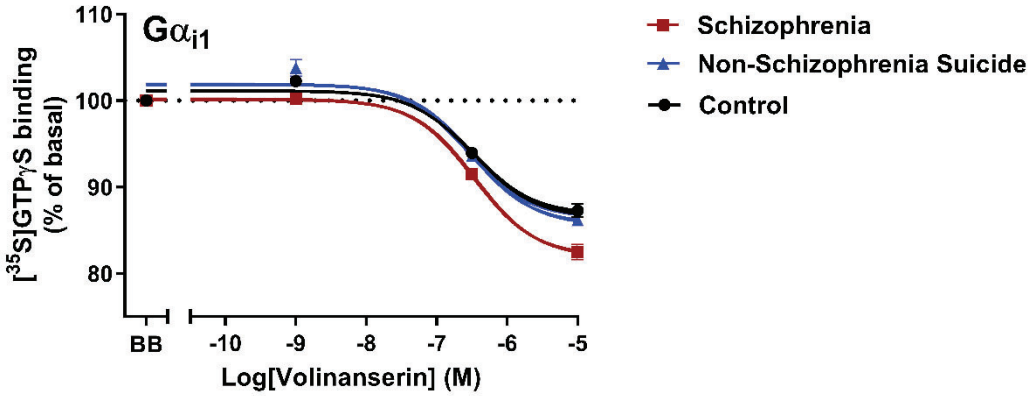



**Figure 4.9**: Concentration-response curves of specific [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins in response to volinanserin in human PFC membranes. The 100% dashed line denotes specific basal [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins (BB). Each point represents mean±SEM value from independent experiments.

**Table 4.10**: Pharmacological parameters of volinanserin concentration-response curves of [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and matched control subjects. $IC_{50}$ values were obtained as antilog of log$IC_{50}$. Imax represents the experimental maximal inhibitory effect induced by volinanserin, and is expressed as percentage of respective basal [$^{35}$S]GTPγS binding value. Values are mean±SEM of n independent subjects. Data were analysed using one-way ANOVA followed by Bonferroni's post-hoc test (**p<0.01 vs control and non-schizophrenia group).

| Group | Basal (fmol/ mg protein) | $IC_{50}$ (nM) | $I_{max}$ (%) | n |
|---|---|---|---|---|
| Schizophrenia | 162±7 | 456±83 | -18±1** | 23 |
| Non-schizophrenia suicide | 175±9 | 359±67 | -14±1 | 13 |
| Control | 159±5 | 341±42 | -13±1 | 23 |

An analysis of covariance (ANCOVA) was further performed to evaluate the potential influence of age at death, PMD and storage time. Sex differences were also evaluated. Any of these variables modified the results and, consistent with previous findings, differences between groups in $I_{max}$ values were maintained (F[2,55]=8.13, p=0.0008).

A complementary statistical comparison between groups was conducted by non-linear curve fitting coanalysis of all individual experiments. The coanalysis of concentration-response curves to volinanserin demonstrated different patterns of schizophrenia respect to control (F[3,178]=14.14, p<0.0001) and non-schizophrenia suicide groups (F[3,138]=8.09, p<0.0001) (**Figure 4.9**). Once statistical differences between curves were obtained, further contrasts were performed to detect whether differences were attributable to changes in $I_{max}$ and/or $IC_{50}$ values between groups. Thus, the curve fitting coanalysis confirmed that schizophrenia subjects showed a higher inhibitory effect of volinanserin ($I_{max}$=-18%, 95%CI 19% to 17%) than control ($I_{max}$=-13%, 95%CI 14% to 12%) (F[1,178]=27.77, p<0.0001) and non-schizophrenia suicide subjects ($I_{max}$=-14%, 95%CI 16% to 13%) (F[1,138]=11.85, p<0.0001). No differences were obtained for $IC_{50}$ values between schizophrenia ($IC_{50}$=355. nM, 95%CI 250.7 nM to 502.6 nM), non-schizophrenia suicide ($IC_{50}$=302.2 nM, 95%CI 186.3 nM to 477.4 nM) and control groups ($IC_{50}$=313.9 nM, 95%CI 208.8 nM to 464.6 nM).

The selectivity of inhibitory effect exerted by volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins was tested by using 5-$HT_{2A}$R neutral antagonist MDL-11,939 at 10 μM. In schizophrenia subjects, volinanserin maximal inhibitory effect on [$^{35}$S]GTPγS binding (-17±1%) was blocked by the antagonist (p<0.0001, t=12.67, df=44) (**Figure 4.10**). Similarly, in non-schizophrenia suicide group, the maximal inhibition promoted by volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (-14±1%) was completely blocked by MDL-11,939 (p<0.0001, t=8.50, df=24) (**Figure 4.10**). As previously described (See 4.1.3),

in control group, the maximal inhibition of [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins induced by volinanserin (-13±1%) was blocked by MDL-11,939 ($p<0.0001$, $t=12.23$, $df=44$) (**Figure 4.10**).

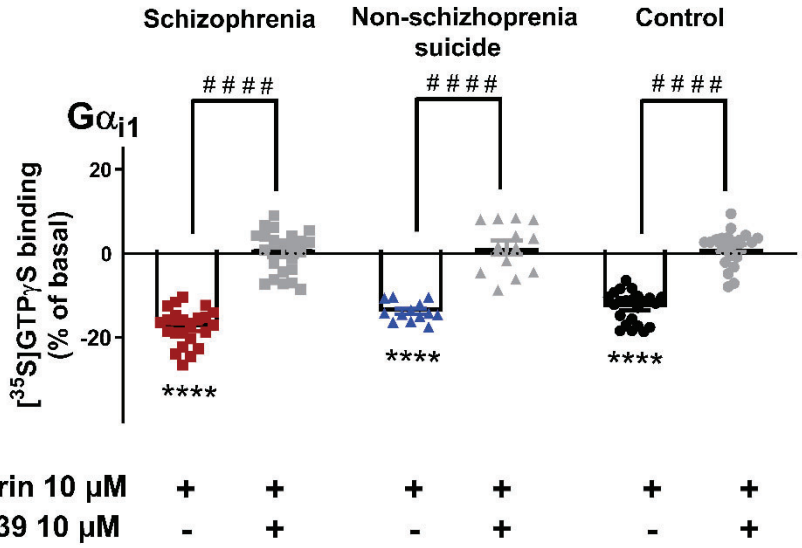



**Figure 4.10**: Antagonism of the inhibitory effect induced by a maximal concentration of volinanserin (10 µM) on specific [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and control subjects. Volinanserin was co-incubated with the selective 5-HT$_{2A}$R antagonist MDL-11,939 (10 µM). Basal values of specific [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins are expressed as 0%, and inhibitory effects on respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (****$p<0.0001$) and two sample t-test to compare volinanserin effects in absence or presence of MDL-11,939. ($^{####}p<0.0001$).

The modulation of [$^{35}$S]GTPγS binding to Gα$_{q/11}$-proteins by the 5-HT$_{2A}$R selective drug volinanserin ($10^{-9}$-$10^{-5}$ M) was also tested in schizophrenia, non-schizophrenia suicide and control groups. As expected, volinanserin displayed concentration-dependent inhibition curves in schizophrenia, non-schizophrenia suicide and control groups (**Figure 4.11**). No differences were observed in potency between groups (schizophrenia: -log$IC_{50}$=6.53±0.1; non-

schizophrenia suicide: -log$IC_{50}$=6.57±0.1; control: -log$IC_{50}$=6.52±0.1 (F[2,56]=1.53, p=0.6617) (**Table 4.11**). Likewise, the inhibitory effect of volinanserin was not different between groups (schizophrenia: $I_{max}$=-16±1%; non-schizophrenia suicide: $I_{max}$=-16±1%; control: $I_{max}$=-15±1%) (F[2,56]=0.6861, p=0.5046).

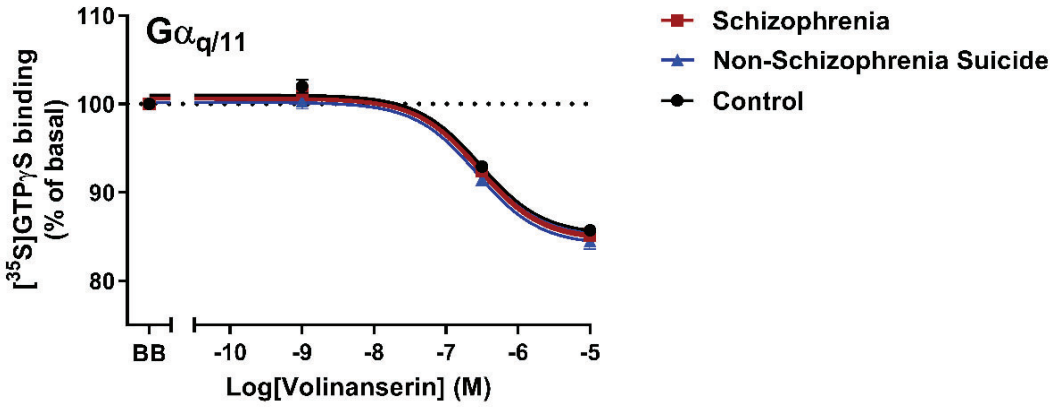



**Figure 4.11**: Concentration-response curves of specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in response to volinanserin in human PFC membranes. The 100% dashed line denotes specific basal [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins (BB). Each point represents mean±SEM value from independent experiments.

**Table 4.11**: Pharmacological parameters of volinanserin concentration-response curves of [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and matched control subjects. $IC_{50}$ values were obtained as antilog of log$IC_{50}$. Imax represents the experimental maximal stimulatory or inhibitory effect induced by volinanserin, and is expressed as percentage of respective basal [$^{35}$S]GTPγS binding value. Values are mean±SEM of n independent subjects. Data were analysed using one-way ANOVA.

| Group | Basal (fmol/ mg protein) | $IC_{50}$ (nM) | $I_{max}$ (%) | n |
|---|---|---|---|---|
| Schizophrenia | 191±9 | 341±54 | -15±1 | 23 |
| Non-schizophrenia suicide | 198±9 | 310±47 | -16±1 | 13 |
| Control | 210±11 | 368±52 | -15±1 | 23 |

A complementary statistical comparison between groups was conducted by non-linear curve fitting coanalysis of all individual experiments. The coanalysis of concentration-response curves to volinanserin demonstrated no different patterns between schizophrenia and control groups (F[3,178]=0.32, p=0.8138) (**Figure 4.11**). In the same way, there was no difference between schizophrenia and non-schizophrenia suicide groups (F[3,138]=0.77, p=0.5103) (**Figure 4.11**). Finally, no changes were found between control and non-schizophrenia suicide group (F[3,138]=2.17, p=0.0948) (**Figure 4.11**).

The selectivity of the inhibitory effect exerted by volinanserin on [$^{35}$S]GTPγS binding to Gα$_{q/11}$-proteins was tested by using the 5-HT$_{2A}$R neutral antagonist MDL-11,939 at 10 μM. In schizophrenia subjects, volinanserin maximal inhibitory effect on [$^{35}$S]GTPγS binding (-15±1%) was also blocked by antagonist ($p<0.0001$, $t=12.49$, $df=44$) (**Figure 4.12**). In non-schizophrenia suicide group, the maximal inhibition promoted by volinanserin on [$^{35}$S]GTPγS binding to Gα$_{q/11}$-proteins (-15±1%), was also completely blocked by MDL-11,939 ($p<0.0001$, $t=10.53$, $df=24$) (**Figure 4.12**). Additionally, in control group, the maximal inhibition of [$^{35}$S]GTPγS binding induced by volinanserin (-14±1%) was also blocked by MDL-11,939 ($p<0.0001$, $t=11.73$, $df=44$) (**Figure 4.12**).

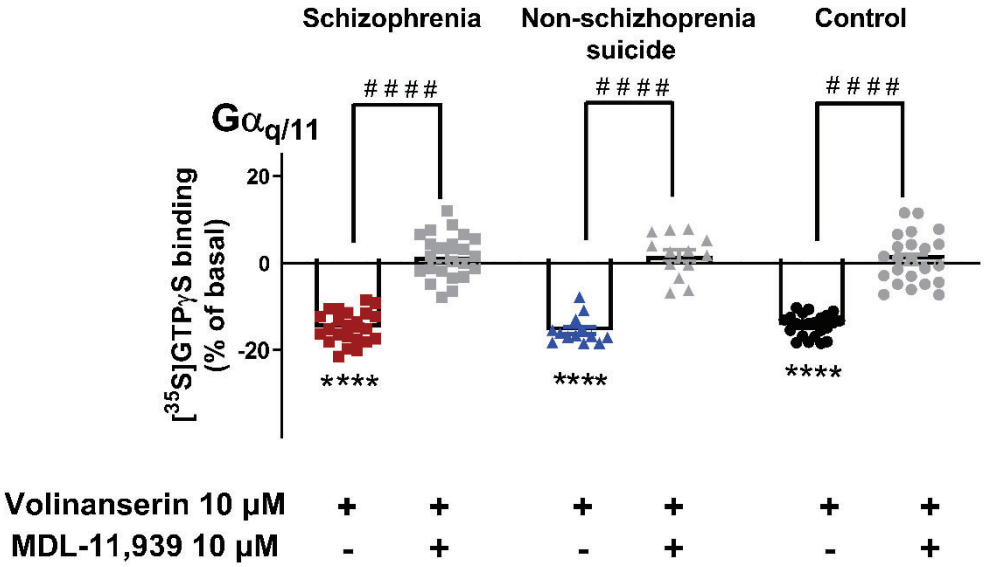



**Figure 4.12**: Antagonism of the inhibitory effect induced by a maximal concentration of volinanserin (10 μM) on specific [$^{35}$S]GTPγS binding to Gα$_{q/11}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and control subjects. Volinanserin was co-incubated with the selective 5-HT$_{2A}$R antagonist MDL-11,939 (10 μM). Basal values of specific [$^{35}$S]GTPγS binding to [$^{35}$S]GTPγS are expressed as 0%, and inhibitory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (****$p<0.0001$) and two sample t-test to compare volinanserin effects in absence or presence of MDL-11,939 ($^{####}p<0.0001$).

### 4.2.4 Modulation of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins by 5-$HT_{2A}$R agonist (±)DOI in post-mortem PFC of schizophrenia subjects, non-schizophrenia suicide subjects and matched controls

In order to confirm previous results (Garcia-Bea et al., 2019), the effect of 5-$HT_{2A}$R selective agonist (±)DOI was evaluated in schizophrenia, non-schizophrenia suicide and control groups at one single concentration (10 μM), which corresponds to a submaximal concentration (**Figure 4.13**). As expected, (±)DOI increased [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in schizophrenia, non-schizophrenia suicide and control groups (**Figure 4.13**). The stimulatory effect of (±)DOI was higher in schizophrenia than in control and non-schizophrenia suicide groups ($F[1,56]=7.45$, $p=0.0014$) (**Table 4.12**) (**Figure 4.13**).

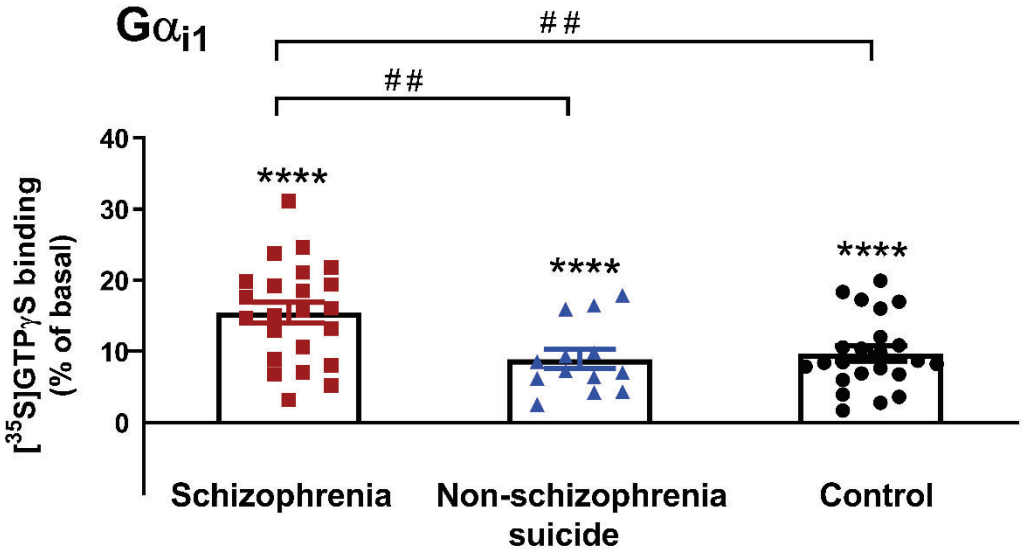



**Figure 4.13**: (±)DOI-induced effect on the specific [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins in human PFC membranes. (±)DOI was added at 10 μM concentration. Basal values of specific [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins are expressed as 0%, and stimulatory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (****p<0.0001) and one-way ANOVA followed by Bonferroni's post-hoc test ($^{\#\#}$p<0.01 vs control and non-schizophrenia group).

**Table 4.12**: Pharmacological parameters of (±)DOI induced-effect at one single concentration (10 μM) on [$^{35}$S]GTPγS binding to Gα$_{i1}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and matched control subjects. $E_{max}$ represents the maximal stimulatory effect induced by (±)DOI, and is expressed as percentage of respective basal [$^{35}$S]GTPγS binding value. Values are mean±SEM of n independent subjects. Data were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test (**p<0.01 vs control and non-schizophrenia group).

| Group | Basal (fmol/ mg protein) | $E_{max}$ (%) | n |
|---|---|---|---|
| Schizophrenia | 162±7 | 15±1** | 23 |
| Non-schizophrenia suicide | 175±9 | 9±1 | 13 |
| Control | 159±5 | 10±1 | 23 |

An analysis of covariance (ANCOVA) was further performed to evaluate the potential influence of age at death, PMD and storage time. Sex differences were also evaluated. Any of these variables modified the results and, consistent with previous findings, the differences between groups in $I_{max}$ values were maintained ($F[2,52]=3.13$, $p<0.05$).

The selectivity of stimulatory effect exerted by (±)DOI on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins was tested by using 5-$HT_{2A}$R neutral antagonist MDL-11,939 at 10 μM. In schizophrenia subjects, (±)DOI-induced maximal stimulatory effect on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (15±1%) was blocked by the antagonist ($p<0.0001$, $t=7.93$, $df=44$) (**Figure 4.14**). In non-schizophrenia suicide group, the maximal stimulation promoted by (±) DOI on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (9±1%) was also completely blocked by MDL-11,939 ($p<0.0003$, $t=4.17$, $df=24$) (**Figure 4.14**). In control group, the maximal stimulation of [$^{35}$S]GTPγS binding induced by (±)DOI (10±1%) was also antagonized by MDL-11,939 ($p<0.0001$, $t=5.86$, $df=44$) (**Figure 4.14**).

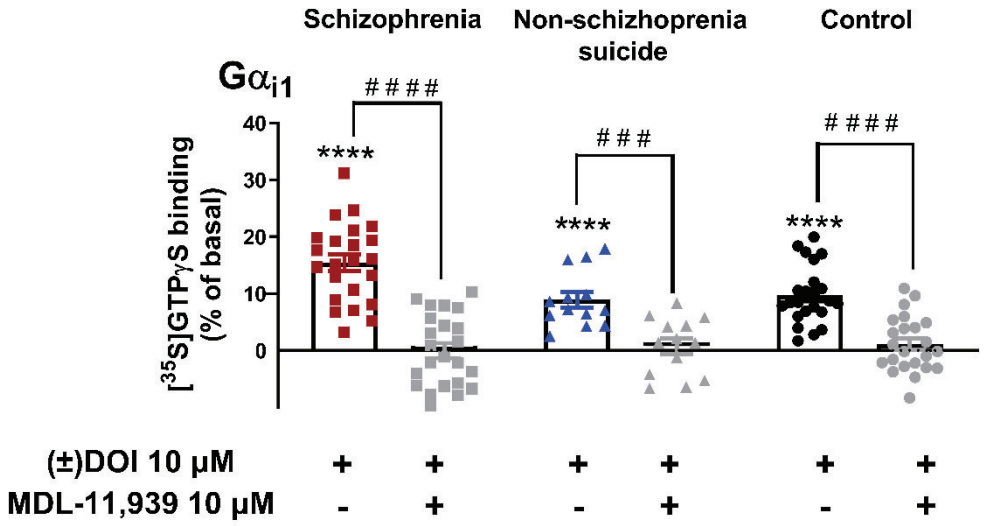



**Figure 4.14**: Antagonism of the stimulatory effect induced by a maximal concentration of (±)DOI (10 µM) on the specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and control subjects. (±)DOI was co-incubated with selective 5-$HT_{2A}$R antagonist MDL-11,939 (10 µM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins are expressed as 0%, and stimulatory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (****p<0.0001) and two sample t-test to compare (±)DOI effects in absence or presence of MDL-11,939 ($^{###}$p<0.001, $^{####}$p<0.0001).

The modulation of [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins by the 5-$HT_{2A}$R selective agonist (±)DOI (10 µM) was also tested in schizophrenia, non-schizophrenia suicide and control groups. As expected, (±)DOI increased the [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in schizophrenia, non-schizophrenia suicide and control groups (**Figure 4.15**). No differences were observed between groups when maximal effects were compared (F[2,55]=2.59, p=0.039) (**Table 4.13**) (**Figure 4.15**).

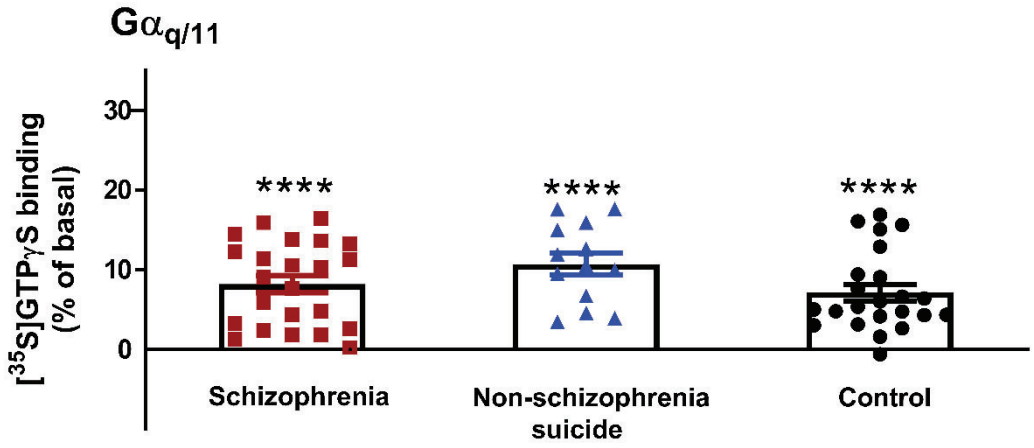



**Figure 4.15**: (±)DOI-induced effect on the specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in human PFC membranes. (±)DOI was added at 10 μM concentration. Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins are expressed as 0%, and stimulatory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (****p<0.0001) and one-way ANOVA followed by Bonferroni's post-hoc test.

**Table 4.13**: Pharmacological parameters of the (±)DOI induced-effect at one single concentration (10 μM) on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and matched control subjects. $E_{max}$ represents the maximal stimulatory effect induced by (±)DOI and is expressed as percentage of respective basal [$^{35}$S]GTPγS binding value**.** Values are mean±SEM of n independent subjects. Data were analysed using one-way ANOVA followed by Bonferroni's post-hoc test.

| Group | Basal (fmol/ mg protein) | Emax (%) | n |
|---|---|---|---|
| Schizophrenia | 191±9 | 8±1 | 23 |
| Non-schizophrenia suicide | 198±9 | 11±1 | 13 |
| Control | 210±11 | 7±1 | 23 |

The selectivity of stimulatory effect exerted by (±)DOI on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was tested by using 5-$HT_{2A}$R neutral antagonist MDL-11,939 at 10 μM. In schizophrenia subjects, (±)DOI maximal stimulatory effect on [$^{35}$S]GTPγS binding (8±1%) was blocked by the antagonist ($p<0.0001$, $t=6.41$, $df=44$) (**Figure 4.16**). In non-schizophrenia suicide group, the maximal stimulation promoted by (±)DOI on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins (11±1%), was completely blocked by MDL-11,939 ($p<0.0002$, $t=4.34$, $df=24$) (**Figure 4.16**). In control group, maximal stimulation of [$^{35}$S]GTPγS binding induced by (±)DOI (7±1%) was also blocked by MDL-11,939 ($p<0.0001$, $t=5.59$, $df=44$) (**Figure 4.16**).

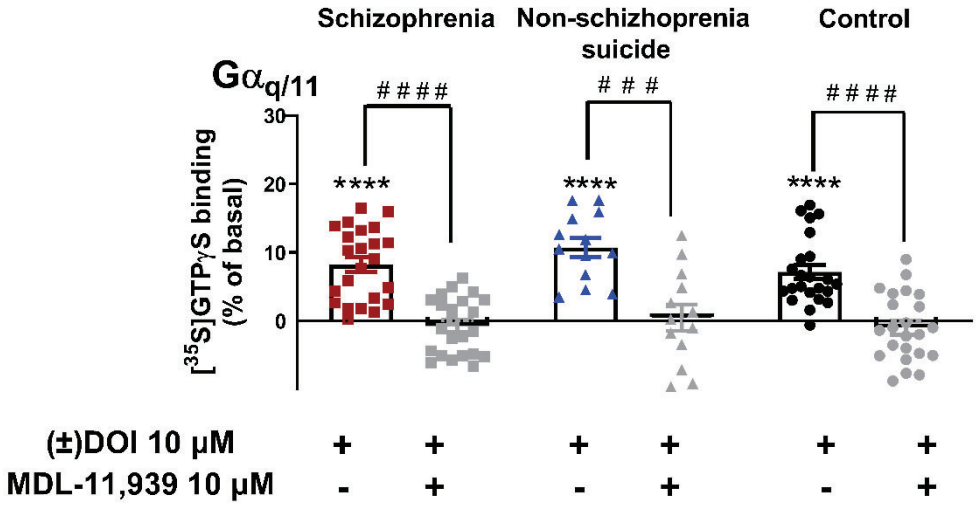



**Figure 4.16**: Antagonism of the stimulatory effect induced by a maximal concentration of (±)DOI (10 μM) on specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in PFC membranes of schizophrenia, non-schizophrenia suicide and control subjects. (±)DOI was co-incubated with selective 5-$HT_{2A}$R antagonist MDL-11,939 (10 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins are expressed as 0%, and stimulatory effects on the respective basal are shown as percentage of basal values. Each point indicates individual values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (****$p<0.0001$) and two sample t-test to compare (±)DOI effects in absence or presence of MDL-11,939 ($^{###}p<0.001$, $^{####}p<0.0001$).

### 4.2.5 Relationship between effects of pimavanserin, volinanserin and (±)DOI on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in post-mortem human PFC

In order to determine the possible relationship between the maximal stimulatory and inhibitory effects exerted on [$^{35}$S]GTPγS binding to each Gα-protein, correlation analyses were carried out between maximal stimulatory effect of (±)DOI (10 µM, assumed as maximal effect induced) and maximal inhibitory effects of pimavanserin and volinanserin (obtained from concentration-response curves).

When considering total number of subjects, a significant positive correlation was found between the maximal stimulatory effect of (±)DOI ($E_{max}$) and the maximal inhibitory effect ($I_{max}$) of pimavanserin exerted on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (r=0.36; n=59; p=0.0049) (**Figure 4.17**).

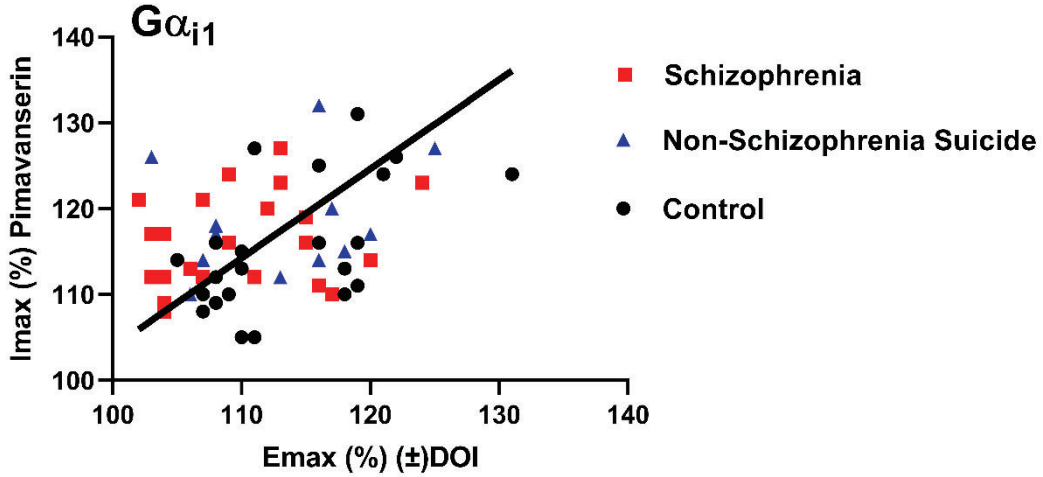



**Figure 4.17:** Linear correlation between the maximal stimulatory effect ($E_{max}$) of (±)DOI and the maximal inhibitory effect ($I_{max}$) of pimavanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in human PFC membranes. The black line represents regression line (Y=1,152*X – 15.24) of the total number of subjects (n=59). Each point indicates individual values of schizophrenia, non-schizophrenia suicide and control subjects.

In the same way, a significant positive correlation was also found between the maximal stimulatory effect of (±)DOI ($E_{max}$) and the maximal inhibitory effect ($I_{max}$) of volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (r=0.29; n=59; p=0.0269) (**Figure 4.18**).

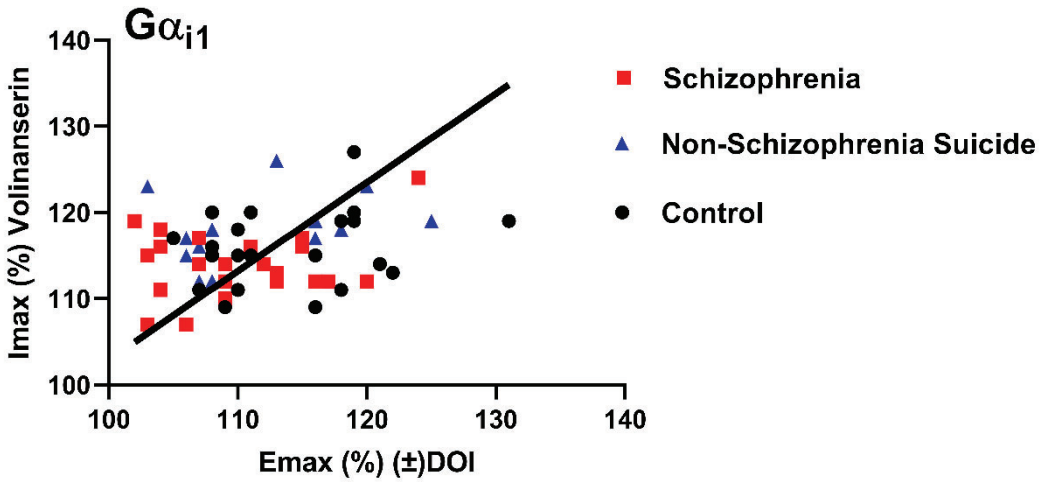



**Figure 4.18:** Linear correlation between the maximal stimulatory effect ($E_{max}$) of (±)DOI and the maximal inhibitory effect ($I_{max}$) of volinanserin on the [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in human PFC membranes. The black line represents the regression line (Y=1.049*X – 4.95) of the total number of subjects (n=59). Each point indicates individual values of schizophrenia, non-schizophrenia suicide and control subjects.

As expected, significant positive correlation was also found between the maximal inhibitory effect of pimavanserin ($I_{max}$) and the maximal inhibitory effect ($I_{max}$) of volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (r=0.32; n=59; p=0.0135) (**Figure 4.19**).

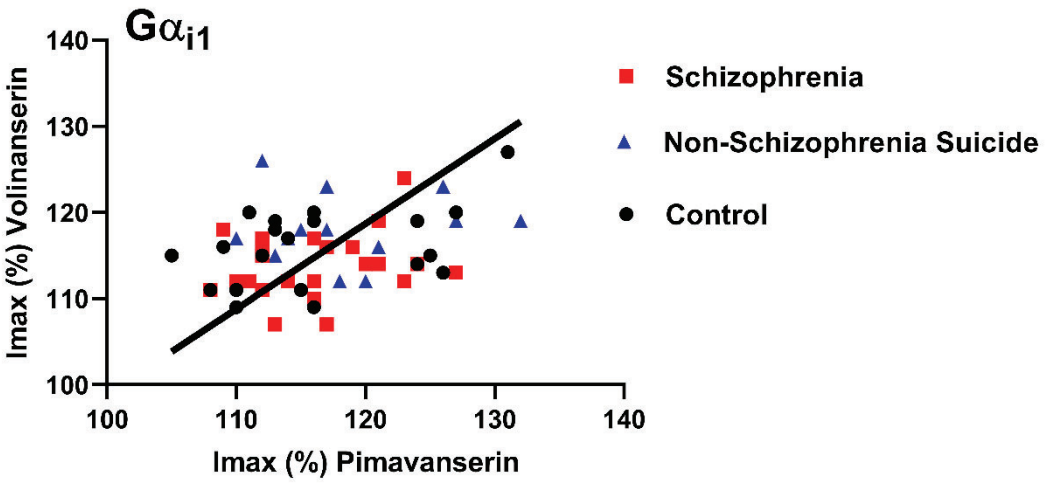



**Figure 4.19:** Linear correlation between the maximal inhibitory effect ($I_{max}$) of pimavanserin and the maximal inhibitory effect ($I_{max}$) of volinanserin on the [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in human PFC membranes. The black line represents the regression line (Y=0.8454*X+ 15.46) of the total number of subjects (n=59). Each point indicates individual values of schizophrenia, non-schizophrenia suicide and control subjects.

The potential relationship between the maximal stimulatory effect of (±)DOI ($E_{max}$) and the maximal inhibitory effect ($I_{max}$) of volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was also tested. In this case, the correlation between both parameters did not show statistical significance (r=0.2008; n=59; p=0.1307) (**Figure 4.20**).

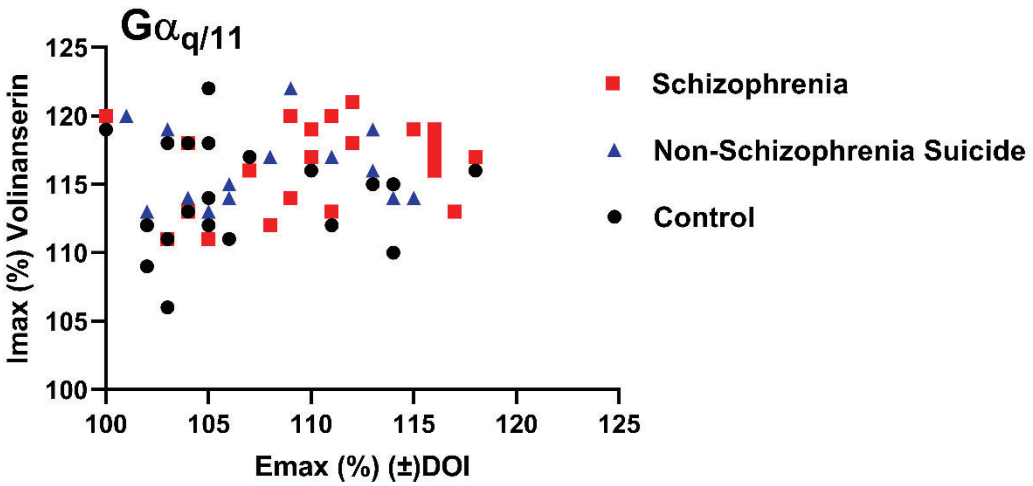



**Figure 4.20:** Linear correlation between the maximal stimulatory effect ($E_{max}$) of (±)DOI and the maximal inhibitory effect ($I_{max}$) of volinanserin on the [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in human PFC membranes. Since no significant linear correlation was obtained, the regression line is not represented. Each point indicates individual values of schizophrenia, non-schizophrenia suicide and control subjects,

### 4.2.6 Summary

In this study, 5-$HT_{2A}$R molecular coupling to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins was evaluated in post-mortem PFC of schizophrenia, non-schizophrenia suicide subjects and matched controls using three different tools with different pharmacological profile. Thus, in order to test the existence of alterations in basal constitutive activity of 5-$HT_{2A}$R in schizophrenia, the inverse agonists pimavanserin and volinanserin were used. As previously seen (4.1.1), pimavanserin decreased [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins with no effect on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins, whereas volinanserin decreased [$^{35}$S]GTPγS binding to both $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein pathways.

The inhibitory effect of pimavanserin and volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins was higher in schizophrenia subjects compared to non-schizophrenia suicide and control subjects. In contrast, the inhibition exerted by volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was similar between schizophrenia subjects, non-schizophrenia suicide subjects and controls.

The stimulatory effect induced by the 5-$HT_{2A}$R agonist (±)DOI on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins was also analysed in the same subjects. (±)DOI displayed enhanced stimulatory effect in 5-$HT_{2A}$R coupling to the $G\alpha_{i1}$-protein pathway in the schizophrenia group compared to schizophrenia suicide and control groups. This finding was not observed in 5-$HT_{2A}$R coupling to the canonical pathway mediated by $G\alpha_{q/11}$-proteins.

These findings demonstrate a selective increase of constitutive activity of 5-$HT_{2A}$R on the $G\alpha_{i1}$-protein-mediated pro-hallucinogenic pathway, which promotes a higher coupling efficiency, in PFC of subjects with schizophrenia. No changes were found in the functional activity of 5-$HT_{2A}$R coupling to $G\alpha_{q/11}$-protein pathway in the same subjects.

## 4.3 Evaluation of immunoreactivity of Gα-protein subtypes in membrane enriched fractions from PFC of post-mortem brain

### 4.3.1 Suitability of antibodies for antibody-capture [$^{35}$S]GTPγS Scintillation Proximity Assay (SPA) and Western Blot

In order to evaluate the specificity of antibodies against each Gα-protein subunit subtype used for SPA assays, Western Blot experiments were carried out. As described in material and methods section **3.5.2**, recombinant proteins and $P_2$ enriched membranes fraction from human, mice and rat cerebral tissue were used.

As shown in **Figure 4.21,** not all tested antibodies labelled a unique band at expected molecular size, albeit the majority of subunits did. Indeed, $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{o}$- and $G\alpha_{q/11}$-proteins showed a single band around 40-45 kDa range in all cases (generally 40 kDa) (**Figure 4.21**). This specificity was obtained in all samples analysed; human, mouse and rat $P_2$ membrane used.

In case of $G\alpha_{s}$-protein, two specific bands at 43 and 46 kDa were obtained, which corresponded to expected isoform described by the manufacturer (**Figure 4.21**).

The use of recombinant proteins demonstrated the absence of cross-reactivity with different Gα subunit subtypes. However, no signal was observed in any of recombinant proteins when they were incubated with the antibody against $G\alpha_{i3}$-protein subtype (**Figure 4.21**).

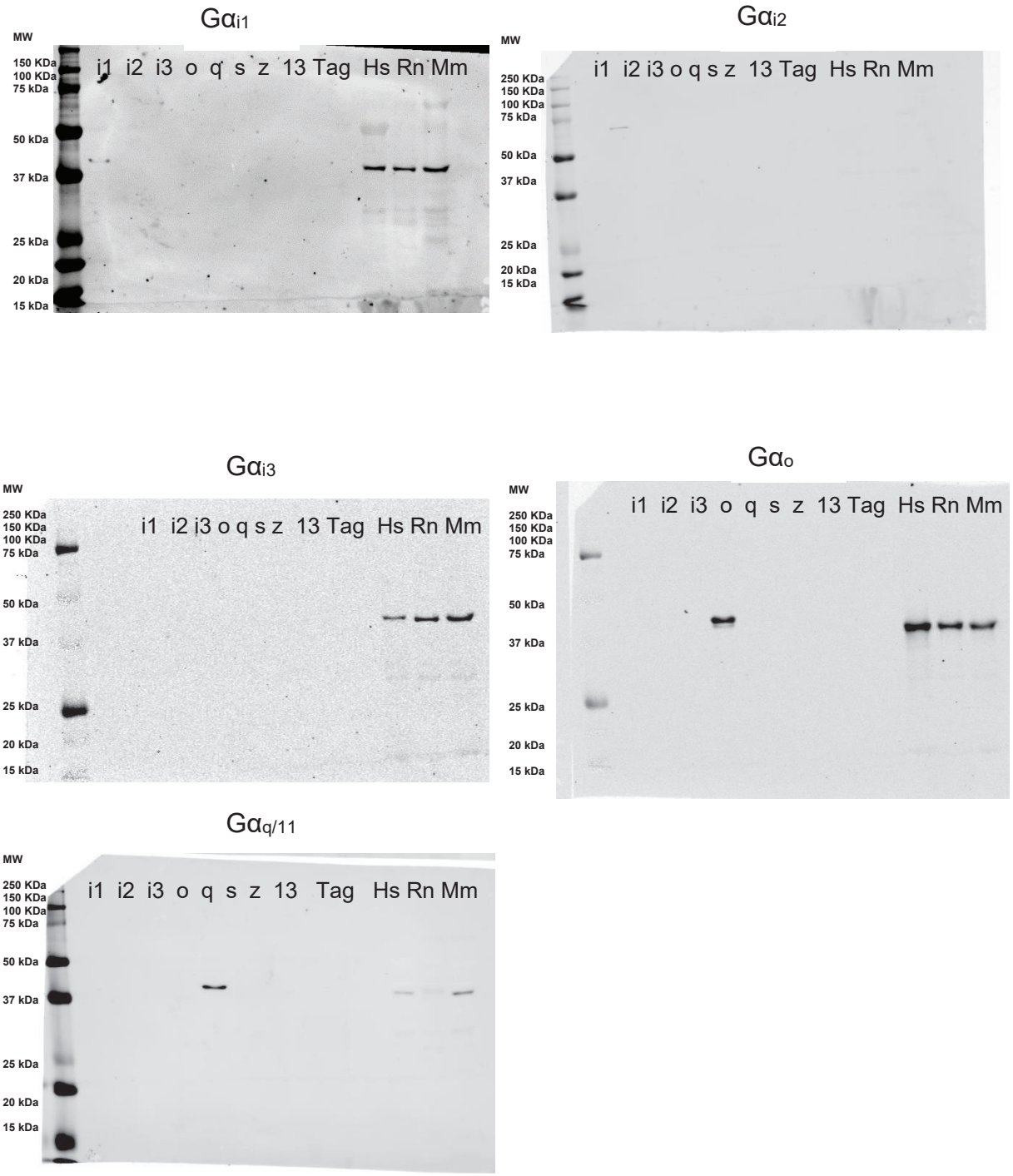



**Figure 4.21**: Representative images of immunoreactive signal obtained by Western Blotting with different antibodies anti Gα-proteins used in antibody-capture [$^{35}$S]GTPγS SPA experiments. $G\alpha_{i1}$ (sc-56536), $G\alpha_{i2}$ (sc-13534), $G\alpha_{i3}$ (sc-365422), $G\alpha_{o}$ (sc-393874), $G\alpha_{q/11}$ (sc-515689) and $G\alpha_{s/olf}$ (sc-377435) using membrane enriched fractions from human PFC (Hs),

mice brain cortex (Mm), rat brain cortex (Rn) and recombinant Gα-protein subunit subtype i1, i2, i3, o, q, s, z and 13.

### 4.3.2 Evaluation of $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein immunoreactivity in post-mortem PFC of schizophrenia subjects, non-schizophrenia suicide subjects, and matched controls

In order to evaluate the influence of variables such as age at death, sex, PMD and storage time on immunoreactivity of both Gα-proteins, linear correlation analyses were performed. Any of these potential variables showed correlation with immunoreactive signal (**Table 4.14**). The absence of relationship was also observed when considered separately the three population groups

**Table 4.14**: Effect of age (years), PMD (hours) and storage time (months) on immunoreactivity of $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins.

| $G\alpha_{i1}$-protein immunoreactivity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Schizophrenia** | | | **Non-schizophrenia suicide** | | | **Control** | | |
| | r | p | n | r | p | n | r | p | n |
| Age (years) | -0.0124 | 0.9541 | 23 | -0.2914 | 0.3581 | 13 | 0.1905 | 0.3727 | 23 |
| PMD (hours) | 0.0636 | 0.7677 | 23 | 0.3429 | 0.2752 | 13 | 0.1056 | 0.6235 | 23 |
| Storage time (months) | 0.3302 | 0.1150 | 23 | 0.1036 | 0.7486 | 13 | 0.4102 | 0.0865 | 23 |
| **$G\alpha_{q/11}$-protein basal binding (fmol/mg protein)** | | | | | | | | | |
| | **Schizophrenia** | | | **Non-schizophrenia suicide** | | | **Control** | | |
| | r | p | n | r | p | n | r | p | n |
| Age (years) | -0.0674 | 0.7544 | 23 | 0.0818 | 0.8005 | 13 | 0.0436 | 0.8395 | 23 |
| PMD (hours) | 0.0846 | 0.6942 | 23 | 0.5096 | 0.0906 | 13 | 0.2459 | 0.2468 | 23 |
| Storage time (months) | 0.4574 | 0.0906 | 23 | -0.2460 | 0.4218 | 13 | 0.0778 | 0.0793 | 23 |

The immunoreacivity of $G\alpha_{i1}$-protein measured in membrane enriched fractions from PFC of schizophrenia (41 kDa band, 93±7%), non-schziophrenia suicide (41 kDa band, 105±6%) and control (41 kDa band, 104±7%) subjects showed no differences between them (F[2,54]=1.34, p=0.3294) (**Figure 4.22.A**). Moreover, when immunoreactivity signal of $G\alpha_{q/11}$-protein was compared between schizophrenia (45 kDa band: 115±4%), non-schizophrenia suicide (45 kDa band, 101±7%) and control subjects (45 kDa band: 118±5%) no differences were found (F[2,54]=2.93, p=0.0612) (**Figure 4.22.B**).

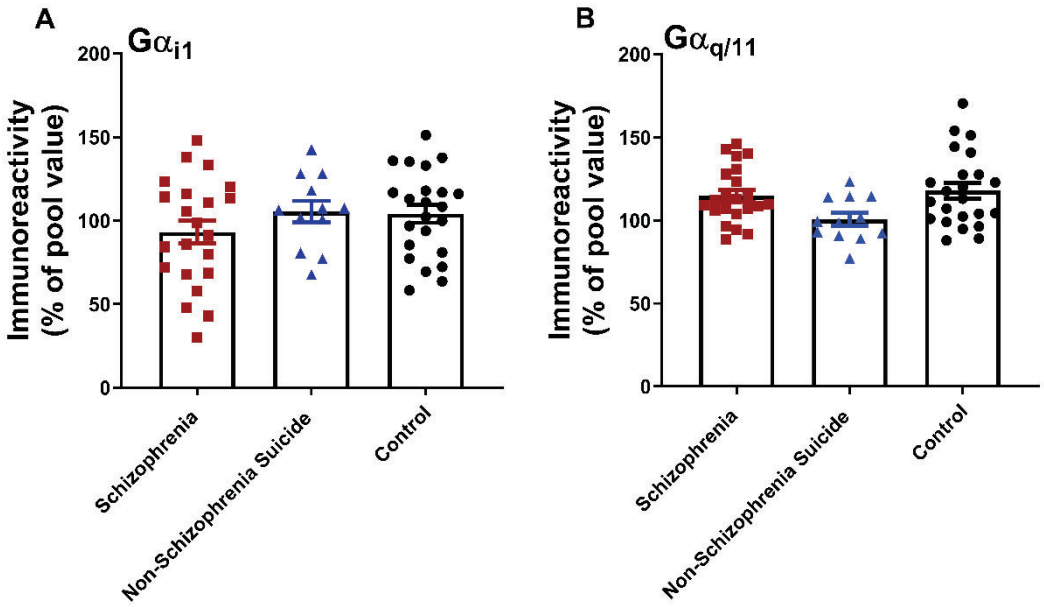



**Figure 4.22**: Graphical representation of cortical levels of $G\alpha_{i1}$- (**A**) and $G\alpha_{q/11}$- (**B**) protein immunoreactivity in PFC membranes of schizophrenia (n=23) (red square), non-schizophrenia suicide (n=13) (blue triangle) and control subjects (n=23) (black circle). The lines represents mean±SEM values of each group.

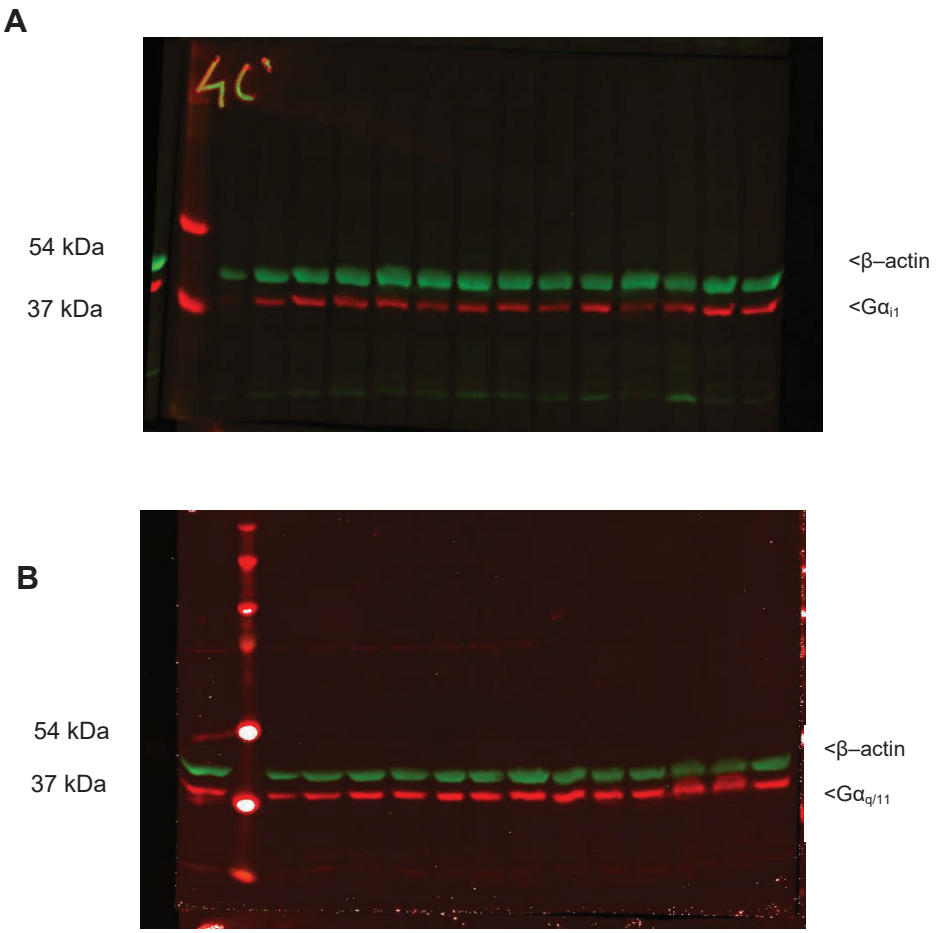



**Figure 4.23**: Representative Odyssey scan of Western Blot depicting immunostaining of $G\alpha_{i1}$ (**A**) and $G\alpha_{q/11}$ (**B**) in red and β-actin in green, in membrane enriched fractions of human PFC from several schizophrenia, control and non-schizophrenia suicide subjects. The molecular weight is displayed on the outer lanes.

### 4.3.3 Summary

In this study we tested the selectivity/specificity of the antibodies for each specific Gα-protein subtype used in antibody-capture [$^{35}$S]GTPγS Scintillation Proximity Assay. The antibodies against $G\alpha_{i1}$-, $G\alpha_{i2}$, $G\alpha_{o}$ and $G\alpha_{q/11}$-protein subtypes showed to be selective. The antibody anti $G\alpha_{i3}$-protein did not provide signal in any of the recombinant proteins.

Secondly, we explored the possible implication of $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein expression in the molecular coupling alterations to 5-$HT_{2A}$R. In order to evaluate the expression of $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein in PFC of subjects with schizophrenia, non-schizophrenia suicide and control subjects, Western Blot experiments were performed (**Figure 4.23**). The analysis of $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in membrane enriched fractions samples revealed no significant differences in protein expression among schizophrenia, non-schizophrenia suicide and control subjects.

## 4.4 Functional selectivity of different antipsychotics in post-mortem human brain PFC. Differential G-protein biased ligand properties at the 5-$HT_{2A}$R

### 4.4.1 Evaluation of maximal effect of different antipsychotic drugs on $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{o}$- and $G\alpha_{q/11}$-protein coupling to 5-$HT_{2A}$Rs in post-mortem human PFC

It is currently recognised that most of ligands previously described as antagonist at GPCRs are, in fact, inverse agonist and this has also been described for antipsychotics (Sullivan et al., 2015). Given the scare information in human post-mortem brain about some pharmacological properties of antipsychotics, such as functional selectivity and intrinsic activity, the responses of specific [$^{35}$S]GTPγS binding to different Gα-proteins after addition of different antipsychotic drugs were investigated.

The functional responses of Gα-proteins to clozapine, olanzapine, risperidone, paliperidone, aripiprazole, quetiapine and haloperidol in human post-mortem PFC were evaluated at one single concentration (10 μM), which corresponds to a concentration promoting submaximal or maximal effect. Furthermore, since antipsychotics possess a multitarget receptor profile, the involvement of 5-$HT_{2A}$R in the modulation of Gα-protein activity was specifically analysed by using neutral antagonist MDL-11,939 at 1 μM concentration to block antipsychotic-induced effects.

Under these experimental conditions, clozapine (-13±1%), olanzapine (-10±3%), risperidone (-20±2%), paliperidone (-13±2%) and haloperidol (-16±2%) induced a statistically significant inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (**Figure 4.24**). Aripiprazole (-3±2%) was unable to modify [$^{35}$S]GTPγS binding, whereas quetiapine promoted a statistically significant stimulation (16±2%) of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (**Figure 4.24**). When 5-$HT_{2A}$R involvement in this effects was evaluated, MDL-11,939 blocked the inhibitory effects of clozapine ($t=4.80$, $df=24$, $p<0.0001$), risperidone ($t=6.28$, $df=22$, $p<0.0001$) and olanzapine ($t=2.26$, $df=6$, $p=0.0646$) although this last antagonist effect did not reach statistical significance (**Figure 4.24**). In contrast, MDL-11,939 did not exert any antagonism either on the stimulatory effect of quetiapine ($t=0.26$, $df=6$, $p=0.8056$) nor on the inhibitory effect of haloperidol ($t=1.23$, $df=9$, $p=0.2395$) (**Figure 4.24**). Finally, MDL-11,939 partially blocked the inhibition exerted by paliperidone ($t=1,67$, $df=9$, $p=0.1296$) (**Figure 4.24**). In conclusion, clozapine, risperidone, olanzapine and probably paliperidone possess inverse agonist properties on 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins (**Table 4.15**).

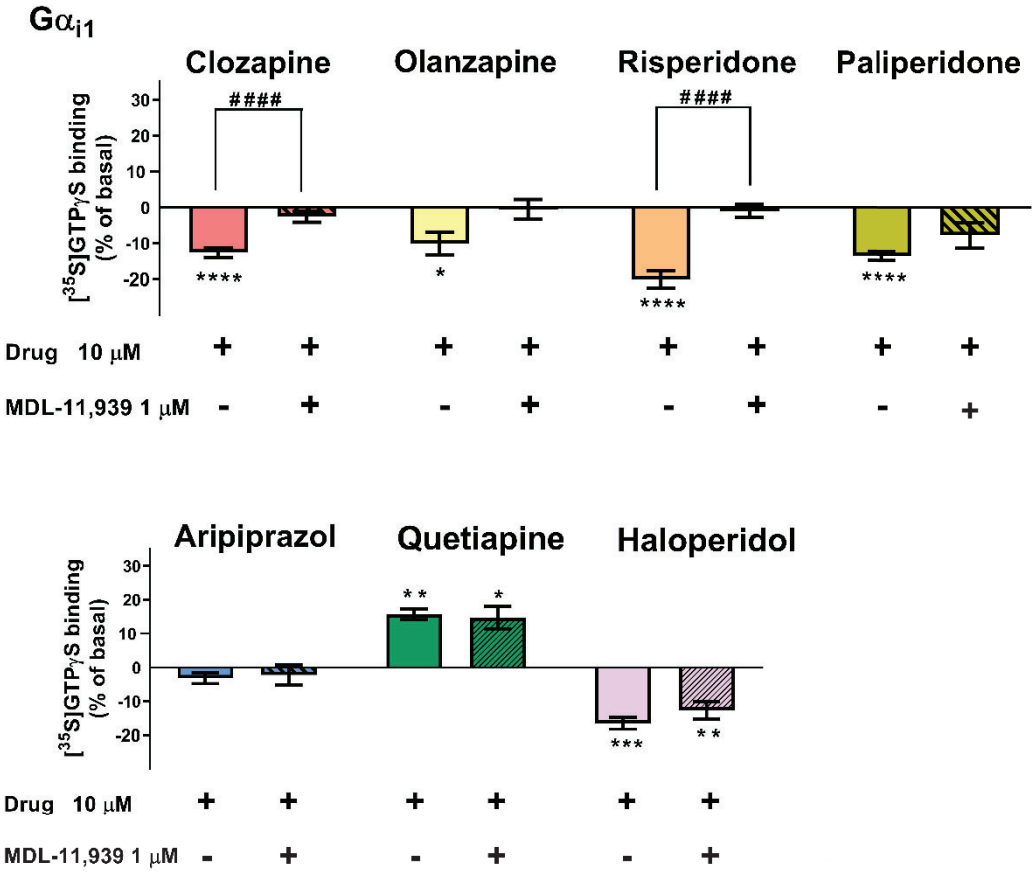



**Figure 4.24**: Representation of the stimulatory or inhibitory effect induced by one single concentration (10 μM) of different antipsychotic drugs (clozapine, olanzapine, risperidone, paliperidone, aripiprazole, quetiapine and haloperidol) on the specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins in human PFC membranes (**Table 4.15**). Antipsychotics were incubated in absence or presence of selective 5-$HT_{2A}$R antagonist MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins are expressed as 0%, and stimulatory or inhibitory effects on respective basal are shown as percentage of basal values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and two sample t-test to compare antipsychotic drug effects in absence or presence of MDL-11,939 ($^{\#\#\#\#}$p<0.0001).

Next, [$^{35}$S]GTPγS binding to $G\alpha_{i2}$-proteins was studied in similar human brain homogenates. Risperidone (-13±4%) and paliperidone (-13±3%) decreased [$^{35}$S]GTPγS binding to $G\alpha_{i2}$-proteins (**Figure 4.25**). Additionally, clozapine (-4±2%), olanzapine (0±2%), aripiprazole (-2±1%), quetiapine (+1±2%) and haloperidol (-1±1%) did not modify basal [$^{35}$S]GTPγS binding to $G\alpha_{i2}$-proteins (**Figure 4.25**). When these antipsychotic drugs were studied in presence of the 5-$HT_{2A}$R neutral antagonist, a full blockage of paliperidone-induced inhibitory effect was observed ($t= 2.97$, $df=7$, $p=0.0208$), suggesting selective 5-$HT_{2A}$R inverse agonist properties for this drug on [$^{35}$S]GTPγS binding to $G\alpha_{i2}$-proteins (**Figure 4.25**). On the other hand, MDL-11,939 was not able to block the inhibitory effect of risperidone ($t= 0.26$, $df=8$, $p=0.8021$) (**Figure 4.25**), indicating that these effect would be mediated via another GPCR (**Table 4.15**).

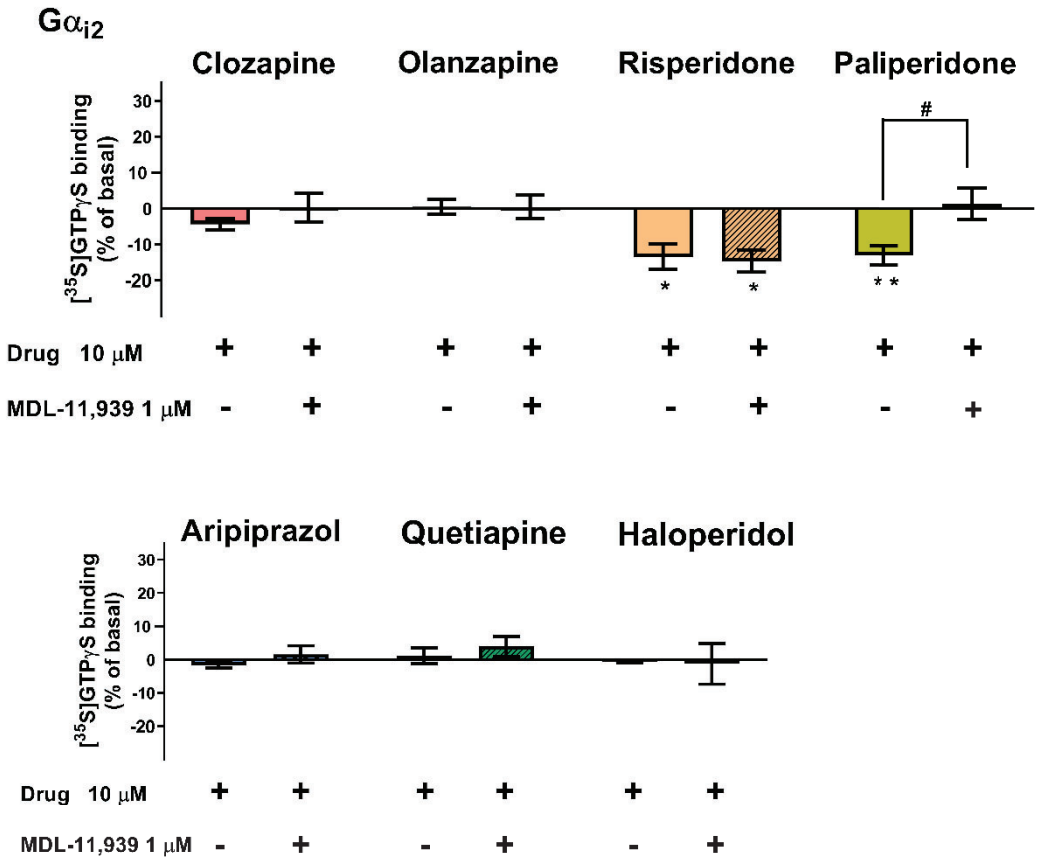



**Figure 4.25**: Representation of the stimulatory or inhibitory effect induced by one single concentration (10 μM) of different antipsychotic drugs (clozapine, olanzapine, risperidone, paliperidone, aripiprazole, quetiapine and haloperidol) on specific [$^{35}$S]GTPγS binding to $G\alpha_{i2}$-proteins in human PFC membranes (**Table 4.15**). Antipsychotics were incubated in absence or presence of selective 5-$HT_{2A}$R antagonist MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{i2}$-proteins are expressed as 0%, and stimulatory or inhibitory effects on respective basal are shown as percentage of basal values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (*$p<0.05$, **$p<0.01$) and two sample t-test to compare antipsychotic drug effects in absence or presence of MDL-11,939 ($^{\#}p<0.05$).

The response of [$^{35}$S]GTPγS binding to Gα$_o$-proteins after addition of different antipsychotics was also analysed. Olanzapine (0±2%), paliperidone (0±1%), quetiapine (5±3%) and haloperidol (-3±1%) were unable to modify basal [$^{35}$S]GTPγS binding to Gα$_o$-protein (**Figure 4.26**). Risperidone was able to decrease this response (-14±3%) while clozapine (8±3%) and aripiprazole (11±3%) increased it (**Figure 4.26**). The inhibitory effects of risperidone was only sensitive to the presence of MDL-11,939 (t=3.17, df=10, p=0.0100) (**Figure 4.26**). Thus, co-incubation of the antagonist with clozapine (t=0.04, df=10, p=0.9699) and aripiprazole (t=0.12, df=10, p=0.9089) did not modify the stimulatory effect exerted by these antipsychotics on [$^{35}$S]GTPγS binding to Gα$_o$-proteins (**Figure 4.26**). These finding suggest that effects induced by risperidone on [$^{35}$S]GTPγS binding of Gα$_o$-protein are mediated by 5-HT$_{2A}$R (**Table 4.15**).

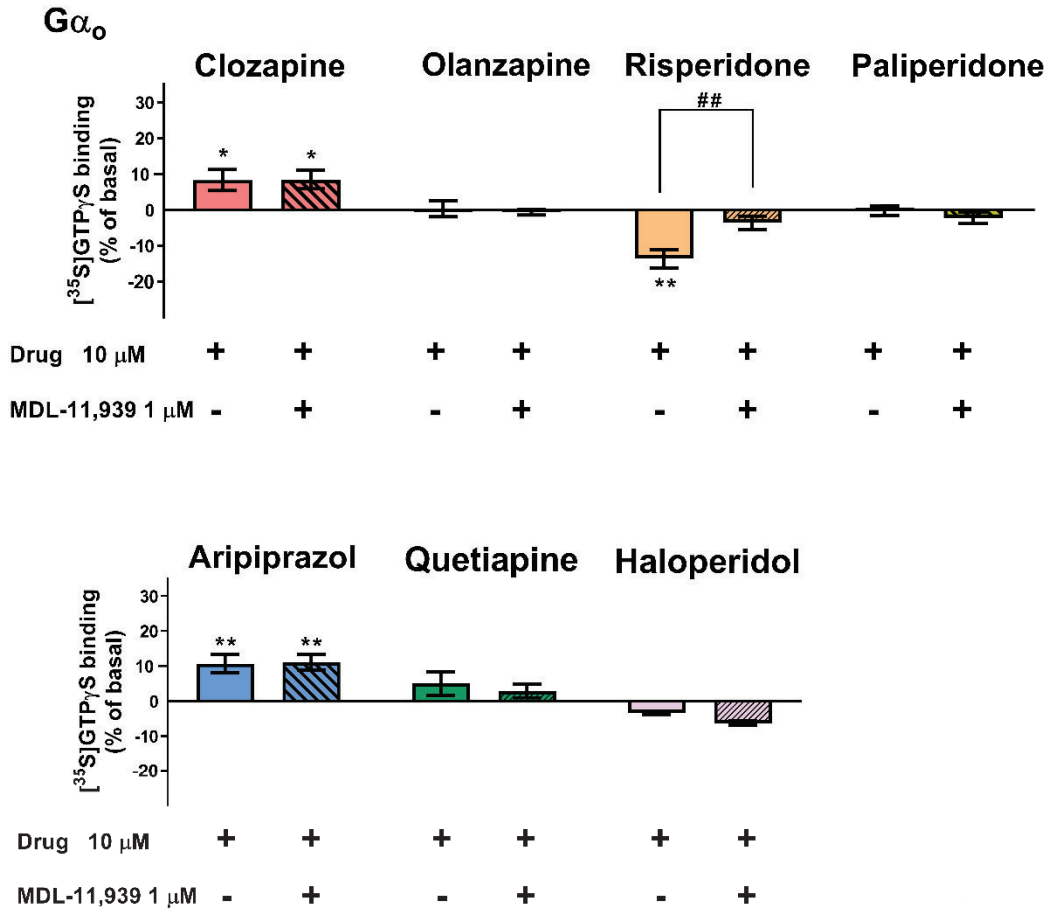



**Figure 4.26**: Representation of the stimulatory or inhibitory effect induced by one single concentration (10 μM) of different antipsychotic drugs (clozapine, olanzapine, risperidone, paliperidone, aripiprazole, quetiapine and haloperidol) on the specific [$^{35}$S]GTPγS binding to Gα$_o$-proteins in human PFC membranes (**Table 4.15**). Antipsychotics were incubated in absence or presence of selective 5-HT$_{2A}$R antagonist MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to Gα$_o$-proteins are expressed as 0%, and stimulatory or inhibitory effects on the respective basal are shown as percentage of basal values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01) and two sample t-test to compare antipsychotic drug effects in absence or presence of MDL-11,939 ($^{\#\#}$p<0.01).

Finally, the modulation of [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins by different antipsychotics was studied in human post-mortem brain membranes. Clozapine increased (18±3%) while risperidone (-13±2%) significantly decreased [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins (**Figure 4.27**). On the other hand, paliperidone (4±3%), olanzapine (-4±5%), aripiprazole (3±2%), quetiapine (-2±3%) and haloperidol (0±1%) had almost a null effect on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins (**Figure 4.27**). MDL-11,939 was not able to block either the stimulatory or inhibitory effects of clozapine ($t=1.71$, $df=24$, $p=0.0997$) and risperidone ($t=1.54$, $df=22$, $p=0.1388$) (**Figure 4.27**). These results point out that effects observed for risperidone and clozapine on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins were not mediated by 5-$HT_{2A}$Rs (**Table 4.15**).

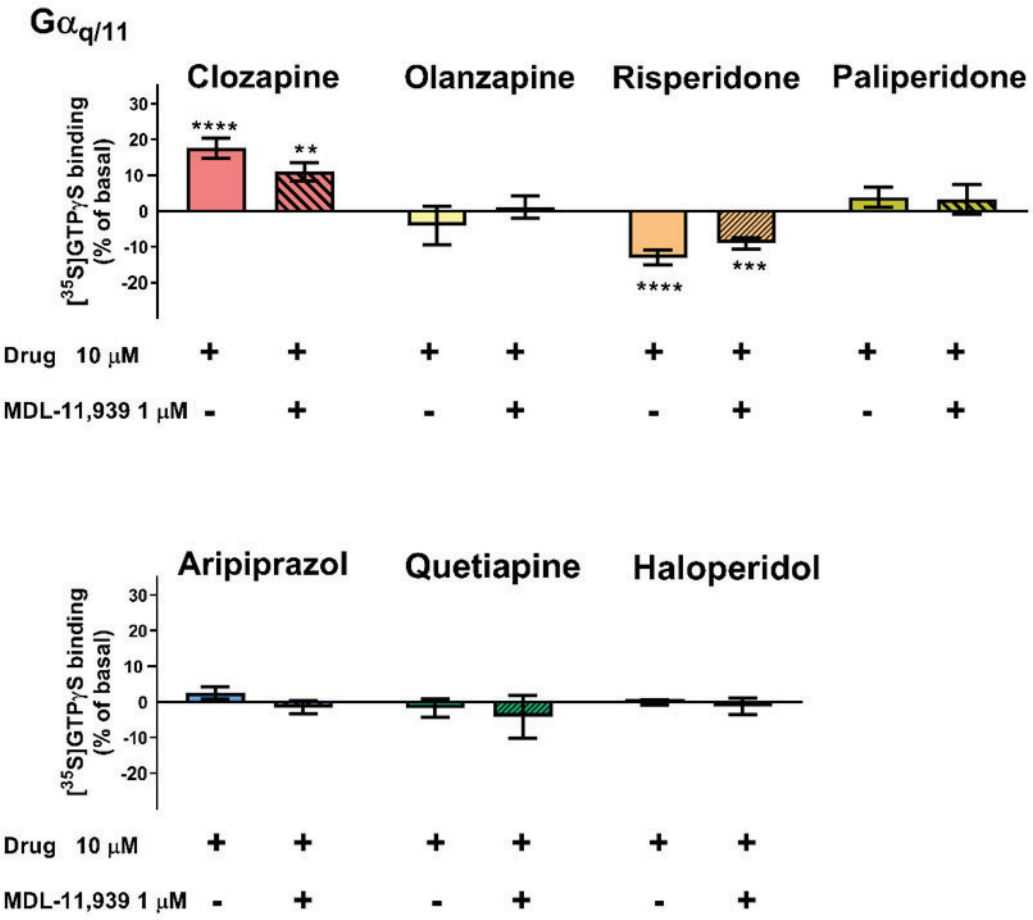



**Figure 4.27**: Representation of the stimulatory or inhibitory effect induced by one single concentration (10 μM) of different antipsychotic drugs (clozapine, olanzapine, risperidone, paliperidone, aripiprazole, quetiapine and haloperidol) on the specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in human PFC membranes (**Table 4.15**). Antipsychotics were incubated in absence or presence of selective 5-$HT_{2A}$R antagonist MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins are expressed as 0%, and stimulatory or inhibitory effects on the respective basal are shown as percentage of basal values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (**p<0.01, ***p<0.001, ****p<0.0001) and two sample t-test to compare antipsychotic drug effects in absence or presence of MDL-11,939.

**Table 4.14**: Numerical results expressed in **Figures 4.23, 4.24, 4.25, 4.26**. Antagonism of the stimulatory or inhibitory effects induced by 10 μM concentration of antipsychotic drugs (clozapine, olanzapine, risperidone, paliperidone, aripiprazole, quetiapine and haloperidol) on specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{o}$- and $G\alpha_{q/11}$-proteins in human PFC membranes. These drugs were incubated in absence and presence of selective 5-$HT_{2A}$R antagonist MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{o}$- and $G\alpha_{q/11}$-proteins are considered as 0%, and stimulatory or inhibitory effects on the respective basal are shown as percentage of basal values. Values represent mean±SEM of percentage values of n independent experiments carried out in duplicate or triplicate. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and two sample t-test to compare antipsychotic drug effects in absence or presence of MDL-11,939 ($^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#\#}$p<0.0001).

| | **$G\alpha_{i1}$-protein** | | **$G\alpha_{i2}$-protein** | |
|---|---|---|---|---|
| | Drug alone 10 μM | Drug + MDL 11,939 1 μM | Drug alone 10 μM | Drug + MDL 11,939 1 μM |
| Clozapine | -13±1**** (n=13) | -3±2$^{\#\#\#\#}$ (n=13) | -4±2 (n=5) | 0±4 (n=5) |
| Olanzapine | -10±3* (n=4) | -1±3 (n=4) | 0±2 (n=5) | 0±3 (n=5) |
| Risperidone | -20±2**** (n=12) | -1±2$^{\#\#\#\#}$ (n=12) | -14±4* (n=5) | -15±3* (n=5) |
| Paliperidone | -13±1**** (n=5) | -8±4 (n=5) | -13±3** (n=5) | 1±4$^{\#}$ (n=5) |
| Aripiprazole | -3±2 (n=6) | -2±3 (n=6) | -2±1 (n=5) | 2±3 (n=5) |
| Quetiapine | 16±2** (n=4) | 15±3 (n=4) | 1±2 (n=5) | 4±3 (n=5) |
| Haloperidol | -16±2*** (n=6) | -13±3** (n=12) | -1±1 (n=2) | -1±6 (n=2) |
| | **$G\alpha_{o}$-protein** | | **$G\alpha_{q/11}$-protein** | |
| | Drug alone 10 μM | Drug + MDL 11,939 1 μM | Drug alone 10 μM | Drug + MDL 11,939 1 μM |
| Clozapine | 8±3* (n=6) | 8±3* (n=6) | 18±3**** (n=13) | 11±3** (n=13) |
| Olanzapine | 0±2 (n=6) | -1±1 (n=6) | -4±5 (n=4) | 1±3 (n=4) |
| Risperidone | -14±3** (n=6) | -4±2$^{\#\#}$ (n=6) | -13±2**** (n=12) | -9±2*** (n=12) |
| Paliperidone | 0±1 (n=6) | -2±2 (n=6) | 4±3 (n=6) | 3±4 (n=6) |
| Aripiprazole | 11±3** (n=6) | 11±2** (n=6) | 3±2 (n=6) | -2±2 (n=6) |
| Quetiapine | 5±1 (n=5) | 3±5 (n=5) | -2±3 (n=4) | -4±6 (n=4) |
| Haloperidol | -3±0 (n=2) | -6±1 (n=2) | 0±1 (n=5) | -1±2 (n=5) |

### 4.4.2 Pharmacological characterization of the effects induced by clozapine and risperidone on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins in post-mortem human PFC

In the previous assays, clozapine and risperidone showed inverse agonist properties on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins attributable to activity on 5-$HT_{2A}$R. Complementary, these two antipsychotic drugs displayed other pharmacological properties on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins, which seem to be 5-$HT_{2A}$R-independent effects and probably mediated by other GPCRs.

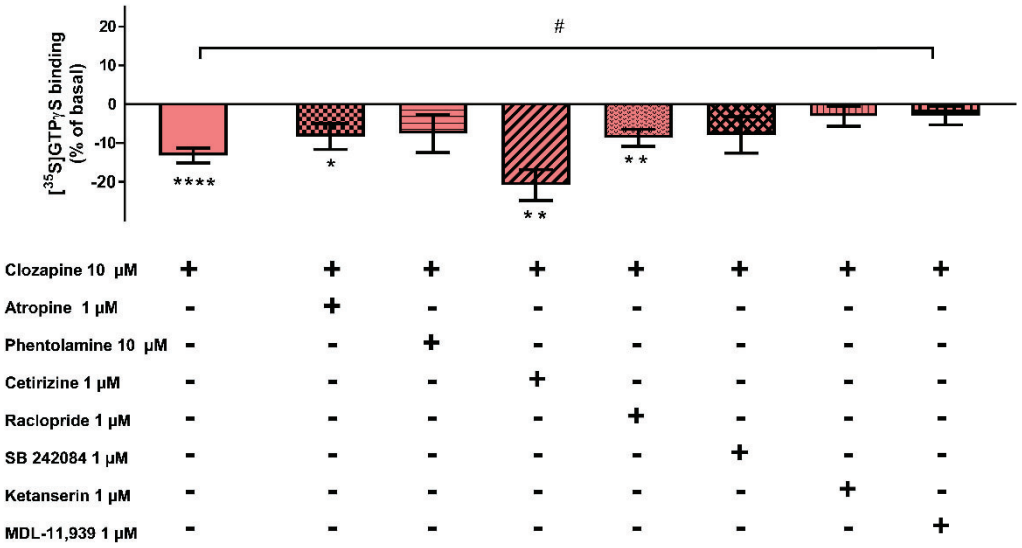
Antipsychotics, in general, have a complex polypharmacology, with appreciable affinities for a number of serotonin (5-$HT_{2A}$, 5-$HT_{2C}$, 5-$HT_6$, 5-$HT_7$, 5-$HT_1$), dopamine ($D_1$, $D_2$, $D_3$, $D_4$, $D_5$) muscarinic ($M_1$, $M_2$, $M_3$, $M_4$, $M_5$), adrenergic ($\alpha_1$- and $\alpha_2$-adrenoceptors), histamine ($H_1$) and other GPCRs (See PDSP Ki database, for further information). In order to characterize the receptors involved in the different effects induced by clozapine and risperidone on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins, antagonist drugs for different GPCRs were used. Atropine (1 μM) was chosen as a non-selective muscarinic antagonist (Kashihara et al., 1992). Phentolamine (10 μM) was selected as α-adrenoceptor antagonist (Devedjian et al., 1994; Waugh et al., 2001).The selective $H_1$R antagonist cetirizine (1 μM) (Moguilevsky et al., 1994) was also used. As risperidone and clozapine show affinity for dopamine $D_2$R family, raclopride (1 μM) was included as a non-selective dopamine $D_2$, $D_3$, $D_4$ receptor antagonist (Sokoloff et al., 1990; Millan et al., 1995). Finally, the differential contribution of 5-$HT_{2A}$R and 5-$HT_{2C}$R was studied by using SB 242084 (1 μM), as selective 5-$HT_{2C}$R antagonist, and ketanserin (1 μM) and MDL-11,939 (1 μM) as 5-$HT_{2A/C}$R and 5-$HT_{2A}$R antagonists, respectively (Knight et al., 2004; Kennet et al., 1994).

### 4.4.2.1 Clozapine

As previously observed, MDL-11,939 antagonized the inhibitory effects exerted by clozapine (-13±2%) ($F[7,76]=4.05$, $p<0.0008$) on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins (**Figure 4.28.A**) (**Table 4.16**). Moreover, ketanserin also induced a blockade of clozapine-induced inhibition that did not reach statistical significance ($p=0.1264$). Other GPCR antagonists did not exert effects on inhibitory activity induced by clozapine on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins. Thus, these results point out that the inhibitory effect of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins promoted by the antipsychotic clozapine is exclusively mediated by 5-$HT_{2A}$R.

On the other hand, atropine and cetirizine at 1 μM blocked the stimulatory effect of clozapine (20±3%) ($F[7,55]=9.07$, $p<0.0001$) on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins (**Figure 4.28.B**) (**Table 4.16**). The rest of antagonists had a null effect on the stimulation induced by clozapine. Cetirizine is a selective antagonist of $H_1$R, which is mostly coupled to $G\alpha_{q/11}$-proteins. Therefore, this receptor could be implicated in the effects of clozapine. Furthermore, $G\alpha_{q/11}$-protein is also the canonical pathway of cholinergic $M_1$R, $M_2$R and $M_3$R. In conclusion, the present results suggested that the stimulatory effects of clozapine on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins could be related to interaction with $H_1$R and/or cholinergic $M_1$R, $M_2$R and $M_3$R.

**$G\alpha_{i1}$ A**




**$G\alpha_{q/11}$ B**

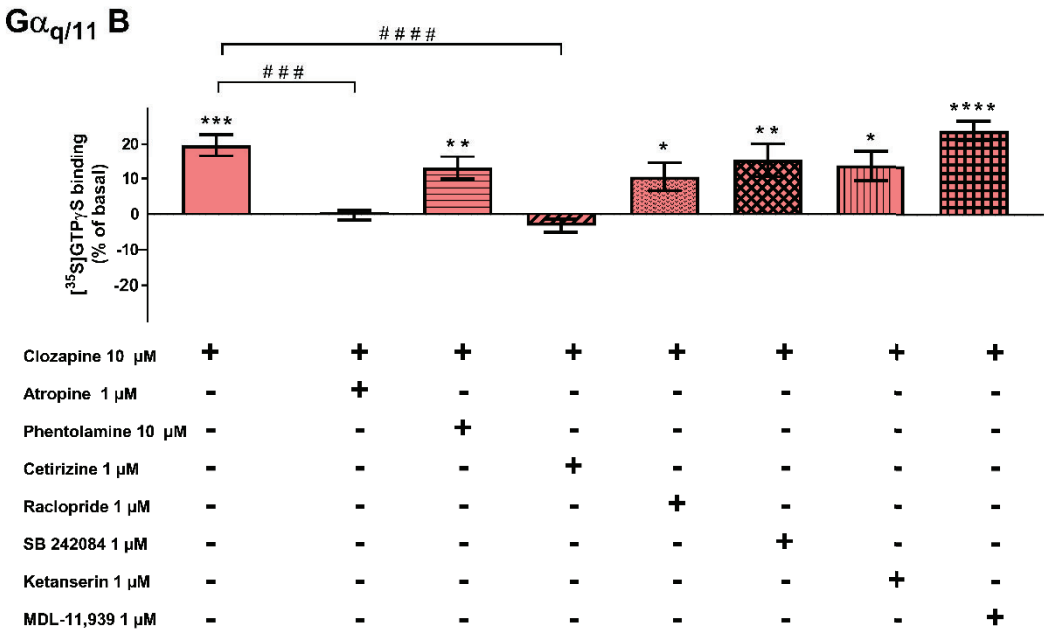


**Figure 4.28**: Representation of the stimulatory or inhibitory effect induced by one single concentration (10 μM) of clozapine on specific [$^{35}$S]GTPγS binding to **Gα$_{i1}$-** (**A**) and **Gα$_{q/11}$-** proteins (**B**) in human PFC membranes (**Table 4.16**). Clozapine was incubated in absence or presence of antagonists atropine (1 μM), phentolamine (10 μM), cetirizine (1 μM), raclopride (1 μM), SB 242084 (1 μM), ketanserin (1 μM) or MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to Gα$_{i1}$- and Gα$_{q/11}$-proteins are expressed as 0%, and stimulatory or inhibitory effects on respective basal are shown as percentage of basal values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and one-way ANOVA followed by Bonferroni´s post-hoc test to compare clozapine effects in absence or presence of the antagonists ($^{\#}$p<0.05, $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001).

**Table 4.16**: Numerical results expressed in **Figure 4.28**. Antagonism of the stimulatory and inhibitory effects induced by 10 μM concentration of clozapine on specific [$^{35}$S]GTPγS binding to Gα$_{i1}$- and Gα$_{q/11}$-proteins in human PFC membranes. Clozapine was incubated in the absence or presence of antagonists atropine (1 μM), phentolamine (10 μM), cetirizine (1 μM), raclopride (1 μM), SB 242084 (1 μM), ketanserin (1 μM) or MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to Gα$_{i1}$- and Gα$_{q/11}$-proteins are considered as 0%, and stimulatory or inhibitory effects on the respective basal are shown as percentage of basal values. Values represent mean±SEM of percentage values of n independent experiments carried out in duplicate or triplicate. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and one-way ANOVA followed by Bonferroni´s post-hoc test to compare clozapine effects in absence or presence of the antagonists ($^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001).

| | **Gα$_{i1}$-protein** | **Gα$_{q/11}$-protein** |
|---|---|---|
| Clozapine 10 μM | -13±2**** (n=11) | 20±3*** (n=8) |
| Clozapine 10 μM + Atropine 1 μM | -8±3* (n=12) | 0±1$^{\#\#\#}$ (n=8) |
| Clozapine 10 μM + Phentolamine 10 μM | -8±2 (n=8) | 13±3**(n=8) |
| Clozapine 10 μM + Cetirizine 1 μM | -20±4** (n=8) | -2±2$^{\#\#\#\#}$ (n=8) |
| Clozapine 10 μM + Raclopride 1 μM | -9±2** (n=8) | 11±4* (n=8) |
| Clozapine 10 μM + SB 242084 1 μM | -8±5 (n=7) | 15±5* (n=8) |
| Clozapine 10 μM + Ketanserin 1 μM | -3±3 (n=8) | 14±4* (n=8) |
| Clozapine 10 μM + MDL-11,939 1 μM | -3±2$^{\#}$ (n=8) | 23±3**** (n=8) |

### 4.4.2.2 Risperidone

The inhibitory effects exerted by risperidone (-16±2%) on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins were antagonized by MDL-11,939, ketanserin and SB 242084 ($F[7,55]=6.17$, $p<0.0001$) (**Figure 4.29.A**) (**Table 4.17**). The rest of the antagonist did not modify the inhibitory effect of risperidone, which discards the involvement of other GPCRs in risperidone effects. In summary, the inhibitory effect of risperidone on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-protein could be mediated via 5-$HT_{2A}$R and/or $5HT_{2C}$R.

Complementary, the inhibitory effect of risperidone (-18±2%) on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was sensitive to phentolamine ($F[7,54]=3.42$, $p=0.0042$) (**Figure 4.29.B**) (**Table 4.17**). The rest of antagonists had a null effect on the inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins promoted by risperidone. These findings suggest that these inhibitory effects of risperidone are mediated by α-adrenoceptors. Given that $G\alpha_{q/11}$-proteins represents the canonical pathway of $\alpha_1$-adrenoceptors, whereas $\alpha_2$-adrenoceptors couple to inhibitory $G\alpha_{i/o}$-proteins, the risperidone effects on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins in human PFC could be probably due to interaction as inverse agonist on $\alpha_1$-adrenoceptors.

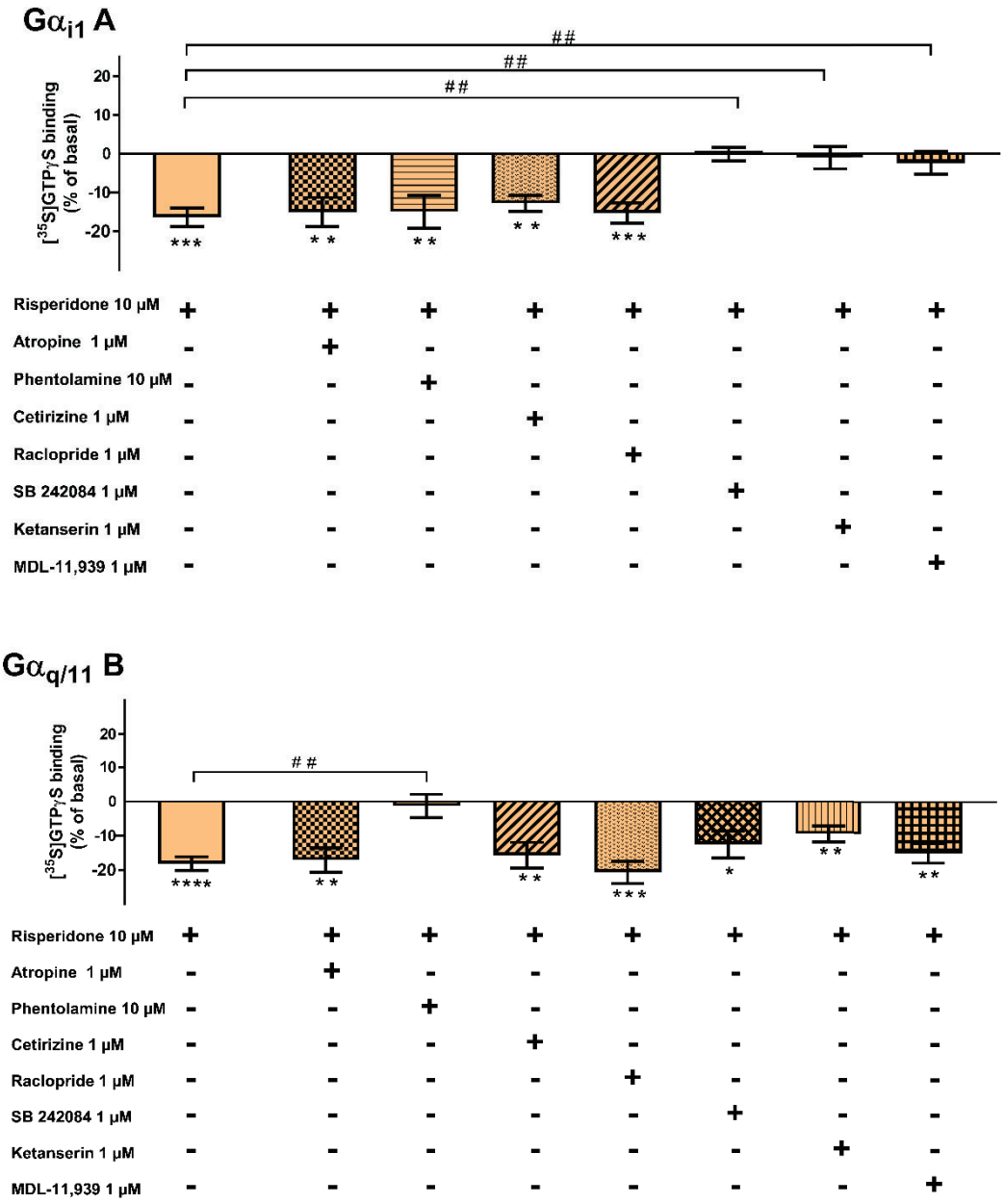

Gαi1 A
[35S]GTPγS binding (% of basal)
20
10
0
-10
-20
##
##
##
***
**
**
**
***
Risperidone 10 µM + + + + + + + +
Atropine 1 µM - + - - - - - -
Phentolamine 10 µM - - + - - - - -
Cetirizine 1 µM - - - + - - - -
Raclopride 1 µM - - - - + - - -
SB 242084 1 µM - - - - - + - -
Ketanserin 1 µM - - - - - - + -
MDL-11,939 1 µM - - - - - - - +
Gαq/11 B
[35S]GTPγS binding (% of basal)
20
10
0
-10
-20
##
****
**
**
***
*
**
**
Risperidone 10 µM + + + + + + + +
Atropine 1 µM - + - - - - - -
Phentolamine 10 µM - - + - - - - -
Cetirizine 1 µM - - - + - - - -
Raclopride 1 µM - - - - + - - -
SB 242084 1 µM - - - - - + - -
Ketanserin 1 µM - - - - - - + -
MDL-11,939 1 µM - - - - - - - +

**Figure 4.29**: Representation of the inhibitory effect induced by one single concentration (10 μM) of risperidone on the specific [$^{35}$S]GTPγS binding to **Gα$_{i1}$-** (**A**) and **Gα$_{q/11}$-**proteins (**B**) in human PFC membranes (**Table 4.17**). Risperidone was incubated in the absence or the presence of the antagonists atropine (1 μM), phentolamine (10 μM), cetirizine (1 μM), raclopride (1 μM), SB 242084 (1 μM), ketanserin (1 μM) or MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to Gα$_{i1}$- and Gα$_{q/11}$-proteins are expressed as 0%, and inhibitory effects on the respective basal are shown as percentage of basal values. Bars represent mean±SEM of percentage values of independent experiments. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and one-way ANOVA followed by Bonferroni´s post-hoc test to compare clozapine effects in absence or presence of the antagonists ($^{\#\#}$p<0.01).

**Table 4.17**: Numerical results expressed in **Figure 4.29**. Antagonism of the inhibitory effects induced by 10 μM concentration of risperidone on the specific [$^{35}$S]GTPγS binding to Gα$_{i1}$- and Gα$_{q/11}$-proteins in human PFC membranes. Risperidone was incubated in the absence or the presence of the antagonists atropine (1 μM), phentolamine (10 μM), cetirizine (1 μM), raclopride (1 μM), SB 242084 (1 μM), ketanserin (1 μM) or MDL-11,939 (1 μM). Basal values of specific [$^{35}$S]GTPγS binding to Gα$_{i1}$- and Gα$_{q/11}$-proteins are considered as 0%, and inhibitory effects on the respective basal are shown as percentage of basal values. Values represent mean±SEM of percentage values of n independent experiments carried out in duplicate or triplicate. Data were analysed using one sample t-test vs basal binding (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and one-way ANOVA followed by Bonferroni´s post-hoc test to compare risperidone effects in absence or presence of the antagonists ($^{\#\#}$p<0.01).

| | **Gα$_{i1}$-protein** | **Gα$_{q/11}$-protein** |
|---|---|---|
| Risperidone 10 μM | -16±2*** (n=8) | -18±2**** (n=8) |
| Risperidone 10 μM + Atropine 1 μM | -15±4** (n=8) | -17±4** (n=7) |
| Risperidone 10 μM + Phentolamine 10 μM | -15±2** (n=7) | -1±3$^{\#\#}$ (n=7) |
| Risperidone 10 μM + Cetirizine 1 μM | -15±3*** (n=8) | -16±4** (n=8) |
| Risperidone 10 μM + Raclopride 1 μM | -13±2*** (n=8) | -20±3*** (n=8) |
| Risperidone 10 μM + SB 242084 1 μM | 0±2$^{\#\#}$ (n=8) | -13±4* (n=8) |
| Risperidone 10 μM + Ketanserin 1 μM | -1±3$^{\#\#}$ (n=8) | -9±2** (n=8) |
| Risperidone 10 μM + MDL-11,939 1 μM | -2±3$^{\#\#}$ (n=8) | -15±3** (n=8) |

### 4.4.3 Summary

The present results support the existence of functional selectivity in the pharmacological profile of antipsychotics activity on different Gα-proteins in human post-mortem brain cortex (**Figure 4.30**). Moreover, these data indicate that some of the evaluated antipsychotic drugs could act as inverse agonist of different GPCRs. Specifically, in relation with all previous findings about 5-$HT_{2A}$R coupling to Gα-proteins, clozapine and risperidone behaved as inverse agonist on the $G\alpha_{i1}$-protein pathway of this receptor, whereas are inactive on the canonical coupling to $G\alpha_{q/11}$-proteins. The finding represents a new view of antipsychotic mechanism of action on the overactivity of 5-$HT_{2A}$R through the hallucinogenic $G\alpha_{i1}$-protein pathway in schizophrenia.

Haloperidol was the only typical or first generation antipsychotic drug included in this study. Haloperidol showed inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins. As expected, this effect was not 5-$HT_{2A}$R-mediated. The pharmacological profile of this effect was not tested further.

Under the present experimental conditions, aripiprazole behaved as agonist for $G\alpha_{o}$-proteins with no effect for $G\alpha_{i1}$-, $G\alpha_{i2}$- and $G\alpha_{q/11}$-proteins. The stimulatory effects was not sensitive to antagonism on 5-$HT_{2A}$R and, as in the case of haloperidol, its pharmacological characterization was not performed.

Quetiapine also showed agonist properties on $G\alpha_{i1}$-proteins, with lack of response to 5-$HT_{2A}$R antagonism. Therefore, the activation of $G\alpha_{i1}$-protein of quetiapine might be mediated by another different GPCR that was not investigated.

The atypical antipsychotic olanzapine inhibited the [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins and did not present any intrinsic activity on other Gα-proteins. Furthermore, this inverse agonism on $G\alpha_{i1}$-proteins was lower than obtained for clozapine and risperidone, suggesting a partial inverse agonist profile. The

olanzapine-mediated inhibition was sensitive to MDL-11,939 blockage, which indicates the involvement of 5-$HT_{2A}$R.

The active metabolite of risperidone, paliperidone showed inhibitory effects on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{i2}$-proteins. The 5-$HT_{2A}$R antagonist MDL-11,939 was able to completely block the inhibitory effects on $G\alpha_{i2}$-proteins, but effects on $G\alpha_{i1}$-proteins were only partially blocked. Overall, paliperidone displays a 5-$HT_{2A}$R inverse agonist profile for coupling to $G\alpha_{i1}$- and $G\alpha_{i2}$-proteins, with null effect on [$^{35}$S]GTPγS binding to $G\alpha_{o}$- and $G\alpha_{q/11}$-proteins. A complementary and unknown inverse agonist activity on $G\alpha_{i1}$ protein by another GPCR could not be discarded, but it was not tested.

Clozapine promoted an inhibition of 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins. In contrast, the response of $G\alpha_{i2}$-protein was unaltered. Clozapine behaved as agonist on [$^{35}$S]GTPγS binding to $G\alpha_{o}$- and $G\alpha_{q/11}$-proteins that did not involve 5-$HT_{2A}$R. Furthermore, the $G\alpha_{q/11}$-protein-mediated agonism of clozapine might represent muscarinic and/or histaminergic receptor activity.

Risperidone exhibited inhibition responses on $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{o}$- and $G\alpha_{q/11}$-protein activity but only the effects on $G\alpha_{i1}$- and $G\alpha_{o}$-proteins were related to a 5-$HT_{2A}$R inverse agonist profile. Finally, the inhibitory effect of risperidone on [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins might be mediated by $\alpha_1$-adrenoceptors.

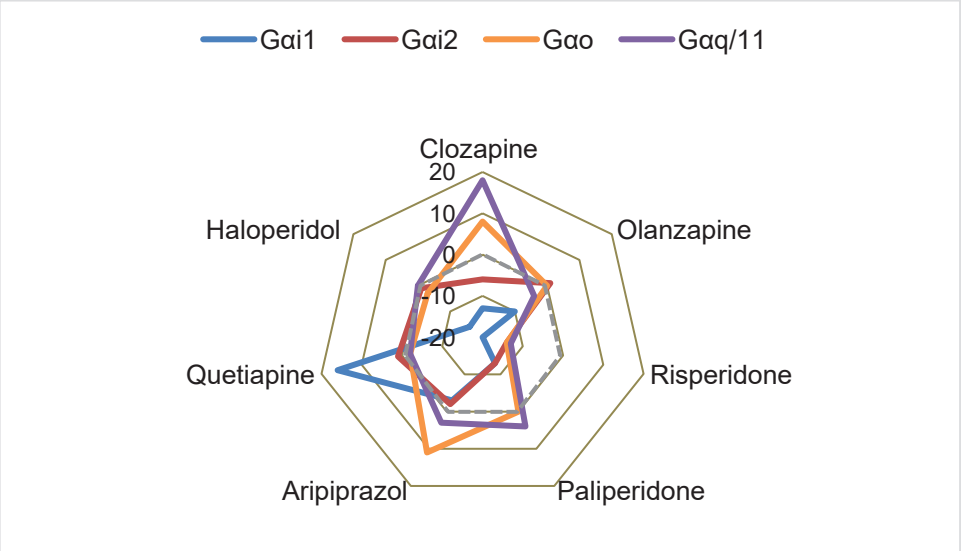



**Figure 4.30**: The differences in activation or inhibition response of $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{io}$- and $G\alpha_{q/11}$-proteins are illustrated in a web plot constructed from data in **table 4.14**. Basal values of specific binding to Gα-proteins are expressed as 0 (dashed line) and inhibitory/stimulatory effects of respective basal are shown as percentage of basal values

# Discussion

During the last decade, important advances have been made in the identification of genes and signalling pathways involved in the risk for psychiatric disorders such as schizophrenia. However, there has been a marked reduction in the pipeline for the development of new psychiatric drugs worldwide, mainly due to the complex causes that underly these disorders (Millan et al., 2015). It is important to note that for CNS diseases like schizophrenia, the underlying aetiology and pathogeny are not known and treatment is often symptomatic. Usually, the mechanism responsible for the therapeutic effects is ascribed to drug properties obtained from tests using *in vitro* systems, which sometimes lack physiological and pathophysiological conditions. This limitation complicates the drug development for CNS disorders.

Constitutive receptor activity, inverse agonism and functional selectivity or biased agonism are concepts in contemporary pharmacology with implications in drug development. Both inverse agonism and functional selectivity are pharmacological properties that need to be considered when drugs are used as medicines or as research tools. In this sense, almost all of the atypical antipsychotic drugs previously thought to be antagonist have been proposed to be inverse agonists at 5-$HT_{2A}$R (Meltzer, 2017). This suggestion is mainly supported by *in vitro* cell culture responses. Arguably, the most obvious use of inverse agonists for therapeutics is to treat diseases that are caused by enhanced constitutive receptor activity. However, until now, there is little evidence about the 5-$HT_{2A}$R constitutive activity in subjects with schizophrenia. A research study addressed to this issue would shed light about the potential therapeutic usefulness of 5-$HT_{2A}$R inverse agonism *vs* antagonism for the disorder. Even so, it could be very difficult to stablish that an *in vivo* effect, or therapeutic effect, of a drug is in fact due to inverse agonism instead of antagonism of endogenous neurotransmitter activity. Consequently, more research in native tissue is needed to better understand

the role of constitutive receptor activity in both physiological functions and disease dysfunctions. The results of these type of studies would help to determine if inverse agonism is important as pharmacotherapeutic property for schizophrenia.

In addition, new ways to assess ligand activity toward multiple signalling pathways in physiologically relevant systems are necessary to generate functional selectivity fingerprints. Development of assays for functional selectivity detection in native tissue might facilitate identification and characterization of ligands that could help to improve treatment of neuropsychiatric diseases with more target selectivity and fewer adverse effects.

In this doctoral Thesis the role of 5-$HT_{2A}$R in schizophrenia was studied. Previous research demonstrated a higher stimulation of $G\alpha_{i1}$-protein but not $G\alpha_{q/11}$-protein by the hallucinogenic drug (±)DOI, through activation of 5-$HT_{2A}$R in post-mortem PFC of schizophrenia subjects. This overactivity seems to reflect conformational modifications more than altered receptor expression, and could represent existence of constitutive activity of 5-$HT_{2A}$R. Therefore, the present study hypothesized the existence of enhanced constitutive activity of 5-$HT_{2A}$R coupled to $G\alpha_{i1}$-protein, the pro-hallucinogenic pathway, in brain of subjects with schizophrenia. Accordingly, the use of selective 5-$HT_{2A}$R inverse agonists, rather than neutral antagonists, with functional selectivity towards $G\alpha_{i1}$-proteins would be a new strategy for schizophrenia treatment. Whether the current antipsychotic drugs with affinity for 5-$HT_{2A}$R meet this pharmacological criterion is unknown. In the next pages, the following arguments revolving these contentions are further discussed.

## 5.1 Evaluation of 5-$HT_{2A}$R functional coupling in human post-mortem brain with different 5-$HT_{2A}$R agonist / antagonist / inverse agonist drugs

GPCR, and among them, the 5-$HT_{2A}$R is a complex system that can activate different signalling pathways, triggering distinct intracellular cascades and leading to diverse physiological effects (López-Giménez & González-Maeso, 2018; Slocum et al., 2021; Pottie & Stove, 2022). These physiological responses depends on both the ligand and the possible GPCR conformations promoted by the reciprocal interaction. Additionally, receptor location and density, oligomerization processes, and other conditions may contribute to the various 5-$HT_{2A}$R functional coupling possibilities. This concept, known as functional selectivity, has been widely described for 5-$HT_{2A}$R and might explain the diverse pharmacological effects observed for different ligands that bind to the same brain cortical 5-$HT_{2A}$R.

In this context, 5-$HT_{2A}$R canonically activate the $G\alpha_{q/11}$-protein but can also activate other Gα-protein-mediated effector pathways (Berg et al., 1998; Kurrasch-Orbaugh et al., 2003a; Kurrasch-Orbaugh et al., 2003b; González-Maeso et al., 2007; Kim et al, 2020). For instance, it has been reported that 5-$HT_{2A}$R hallucinogenic agonist drugs activate the 5-$HT_{2A}$R coupling to $G\alpha_{q/11}$-proteins, as well as $G\alpha_{i/o}$-protein subtypes, while non-hallucinogenic 5-$HT_{2A}$R agonist drugs only activate the 5-$HT_{2A}$R-mediated triggering of $G\alpha_{q/11}$-protein signalling pathway (González-Maeso et al., 2003; González-Maeso et al., 2007; Muneta-Arrate et al., 2020). Moreover, different studies also support that 5-$HT_{2A}$R hallucinogenic agonists display a varied functional selectivity for Gα-protein and β-arrestin recruitment (Kim et al., 2020; Cao et al., 2022).

The present study compares the pattern of 5-$HT_{2A}$R coupling to different Gα-proteins in human post-mortem PFC triggered by different ligands. The [$^{35}$S]GTPγS SPA binding assay combined with specific antibodies against different Gα-protein subunits was used as the most suitable methodological

tool to discriminate the coupling between the different Gα-proteins in native tissue as the human PFC. Ligands were selected among those previously proposed as antagonist or inverse agonists of the 5-$HT_{2A}$R.

The study of Gα-protein coupling modulation by using different 5-$HT_{2A}$R ligands demonstrates that 5-$HT_{2A}$R displays functional coupling not only to the canonical $G\alpha_{q/11}$-protein pathway but also to different inhibitory $G\alpha_{i/o}$-proteins in human PFC. Among these inhibitory $G\alpha_{i/o}$-proteins, the $G\alpha_{i1}$-protein subtype was the most prominent in the receptor-mediated responses to the antagonist / inverse agonist drugs.

Previous results of the research group demonstrated that hallucinogenic 5-$HT_{2A}$R agonists such as (±)DOI, 1-(4-Bromo-2,5-dimethoxyphenyl)-2-aminopropane (DOB), (3-bromo-2,5-dimethoxy-7-bicyclo[4.2.0]octa-1(6),2,4-trienyl)methanamine hydrobromide (TCB-2) and LSD are able to activate different inhibitory $G\alpha_{i/o}$-proteins and $G\alpha_{q/11}$-proteins in human PFC (Miranda-Azpiazu et al., 2013). In contrast, in the same tissue, the non-hallucinogenic 5-$HT_{2A}$R agonist lisuride promoted exclusively activation of the canonical $G\alpha_{q/11}$-protein pathway (Miranda-Azpiazu et al, 2013). The present study complements these previous findings by focusing on ligands with putative neutral or inhibitory effects on 5-$HT_{2A}$R coupling to the different Gα-protein subtypes. The supersensitive 5-$HT_{2A}$R coupling to the pro-hallucinogenic $G\alpha_{i1}$-protein-mediated pathway in schizophrenia, without alterations in the canonical $G\alpha_{q/11}$-protein pathway, (García-Bea et al., 2019; Odagaki et al., 2021) turns biased antagonist / inverse agonist ligands into interesting tools for therapeutics of the disorder.

Previous studies have compared two distinct cellular signal transduction pathways of 5-$HT_{2A}$R, PLC-mediated IP accumulation and PLA2-mediated AA release (Berg et al., 1998; Kurrasch-Orbaugh et al., 2003a; Kurrasch-Orbaugh et al., 2003b; Martí-Solano et al., 2015). Literature supports the involvement of $G\alpha_{q/11}$-proteins in the regulation of IP concentrations and, for many years, this

measurement have been used to identify $G\alpha_{q/11}$-protein signalling pathway activity (Mizuno & Itoh, 2009). However, little is known about the activation of PLA2, and subsequently release of AA. Kurrasch-Orbaugh et al., concluded that 5-$HT_{2A}$R could activate PLA2 signalling through a complex signalling mechanism involving both $G\alpha_{i/o}$ associated $G\beta\gamma$-mediated ERK-1,2 activation and $G\alpha_{12/13}$-coupled Rho mediated p38 activation (Kurrasch-Orbaugh et al., 2003a). AA release regulation has been studied in many different laboratories using a variety of different cellular systems.

More recently, Kim et al. performed BRET-based assays to study 5-$HT_{2A}$R interactions with different proteins, including Gα-proteins and β-arrestins, (Olsen et al., 2020). This study revealed that, in presence of different agonist, 5-$HT_{2A}$R coupled robustly to $G\alpha_q$-family members (Kim et al., 2020). However, 5-$HT_{2A}$R coupled minimally to conventional $G\alpha_{i/o}$- or $G\alpha_s$-protein family members, with the exception of $G\alpha_z$-proteins. The report also confirmed the selective interaction between β-arrestins and 5-$HT_{2A}$R, which seems to be implicated in the hallucinogenic effects of 5-$HT_{2A}$R agonists (Kim et al., 2020; Rodriguiz et al., 2021).

In contrast, other authors (Inoue et al., 2019; Avet et al., 2022) using different BRET approaches concluded that 5-$HT_{2A}$R can couple efficiently with all the tested Gα-proteins in presence of 5-HT. These contradictory results could be due to differences in the modified proteins used to measure the 5-$HT_{2A}$R coupling by BRET.

Herein, in order to characterize the functional coupling of 5-$HT_{2A}$R, different drugs (altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin, ketanserin, eplivanserin and MDL-11,939) were chosen. These drugs have been described as selective 5-$HT_{2A}$R antagonists and in some cases as inverse agonists (Jones et al., 2020; Casey et al., 2022). Some of them (pimavanserin, ritanserin, ketanserin) show also important affinity for 5-$HT_{2C}$R (Fiorella et al., 1995; Knight et al., 2004).

Altanserin, pimavanserin, nelotanserin, ritanserin, volinanserin and eplivanserin displayed concentration-dependent inhibitory responses on the $G\alpha_{i1}$-protein activity. This fact points out the existence of basal constitutive activity to $G\alpha_{i1}$-proteins, which could be reduced by the drugs. In contrast, ketanserin and MDL-11,939 had a null effect in the [$^{35}$S]GTPγS basal activity of $G\alpha_{i1}$-proteins, behaving as neutral antagonists.

The inhibitory effects of altanserin, pimavanserin and volinanserin on $G\alpha_{i1}$-proteins were sensitive to selective 5-$HT_{2A}$R antagonists and absent in 5-$HT_{2A}R^{(-/-)}$ mice. These results confirmed the involvement of 5-$HT_{2A}$R and the inverse agonist properties of these drugs. In contrast, nelotanserin, ritanserin and eplivanserin seemed to decrease $G\alpha_{i1}$-protein basal activity by a GPCR other than 5-$HT_{2A}$R.

On the other side, when the canonical pathway was studied, ketanserin increased the [$^{35}$S]GTPγS basal binding to $G\alpha_{q/11}$-proteins, displaying agonist properties. Conversely, altanserin, pimavanserin and MDL-11,939 showed a null effect in the [$^{35}$S]GTPγS basal binding for $G\alpha_{q/11}$-proteins. Finally, ritanserin, volinanserin and eplivanserin showed concentration-dependent inhibitory properties for the $G\alpha_{q/11}$-protein signalling pathway. When selective antagonists and 5-$HT_{2A}R^{(-/-)}$ mice were used in similar assays, only volinanserin inhibitory and ketanserin stimulatory effects were absent. Therefore, volinaserin represented an inverse agonist drug on 5-$HT_{2A}$R coupling to $G\alpha_{q/11}$-proteins and confirmed the existence of basal constitutive activity in this pathway. The inhibitory effects of ritanserin and eplivanserin on $G\alpha_{q/11}$-proteins would probably represent a pharmacological effect mediated by another GPCR.

Therefore, it might be concluded that different ligands have the ability to display functional selectivity properties on the 5-$HT_{2A}$R coupling to Gα-proteins in human PFC, as well as, in some cases, the ability to decrease the [$^{35}$S]GTPγS

basal binding to the different Gα-proteins with inverse agonist properties. The findings in brain mouse seem to reproduce the results in human brain.

Interestingly, MDL-11,939 was the only drug that behaved as neutral antagonist for the different Gα-protein pathways in human and mouse brain. Therefore, MDL-11,939 was chosen as preferred 5-$HT_{2A}$R antagonist tool for the subsequent studies.

In the same way, volinanserin inhibited the [$^{35}$S]GTPγS basal binding to $G\alpha_{i1}$- and also, with lower efficacy, to $G\alpha_{q/11}$-proteins. Therefore, it represents the only 5-$HT_{2A}$R drug, among studied, with inverse agonist properties on both pathways in human PFC. This pharmacological characteristic represents an advantage because it would allow to evaluate inverse agonism properties on different pathways with an unique tool.

Although previous studies described altanserin and pimavanserin as inverse agonist in human post-mortem brain, it should be taken into account that those studies were performed with classical [$^{35}$S]GTPγS binding assays (Diez-Alarcia et al., 2019). These assays do not discriminate between Gα-proteins and mainly represent receptor coupling to $G\alpha_{i/o}$-proteins (Diez-Alarcia et al., 2021).

Ritanserin was the only drug decreasing the basal binding of all the studied Gα-proteins, indicating an unbiased inverse agonist pharmacological profile. However, this pharmacological profile does not represent 5-$HT_{2A}$R-mediated activity, at least by $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins. In addition to 5-$HT_{2A}$R, ritanserin displays important affinity for 5-$HT_{2C}$R, $H_1$R, $D_2$R, and $\alpha_2$-adrenergic receptor receptors (Leysen et al., 1985).

Surprisingly, ketanserin showed agonist properties on receptor coupling to $G\alpha_{q/11}$-proteins, with lack of response on other Gα-proteins. This agonist effect was mediated via 5-$HT_{2A}$R, as it was blocked by MDL-11,939 and absent in 5-$HT_{2A}R^{(-/-)}$ animals. Therefore, although ketanserin has been used for a long

time as reference antagonist, in fact, this drug results to have partial biased agonist properties.

Previous studies in mouse somatosensory cortex and cells expressing 5-$HT_{2A}$R demonstrated that hallucinogenic and non-hallucinogenic 5-$HT_{2A}$R agonist are able to activate different transcriptome responses (González-Maeso et al., 2003). Together with non-selective c-fos activation by 5-$HT_{2A}$R agonists, the increase of erg-1 and erg-2 mRNA expression in presence of agonist was sensitive to PTX, confirming the involvement of $G\alpha_{i/o}$-proteins in the coupling of 5-$HT_{2A}$R (González-Maeso et al., 2007; Karaki et al., 2014).

Previous studies in human and mouse brain showed that the higher stimulatory response of inhibitory $G\alpha_{i/o}$-proteins in presence of the agonist (±)DOI was obtained with the $G\alpha_{i1}$-protein subtype (Miranda-Azpiazu et al., 2013; Ibarra-Lecue et al., 2018; Garcia-Bea et al., 2019). Here, the receptor coupling to the different Gα-proteins was evaluated again because new pharmacological tools were tested. Different responses to the diverse Gα-proteins were obtained except unability to test $G\alpha_{i3}$-protein involvement. After Western Blot experiments using commercial recombinant Gα-proteins to characterize the antibodies, it was realised that the $G\alpha_{i3}$-antibody was not able to detect any protein.The present findings related to modulation of inhibitory $G\alpha_{i/o}$-proteins mediated by inverse agonists were consistent with previously reported stimulatory effects of agonist drugs. Thus, the $G\alpha_{i1}$-protein subtype displayed the highest activity in response to 5-$HT_{2A}$R drugs. The results strength the role of $G\alpha_{i1}$-protein coupling to 5-$HT_{2A}$R, even when different monoclonal antibodies are selected for immunoprecipitation of the protein in SPA assays (Diez-Alarcia et al., 2021).

## 5.2 Constitutively active 5-$HT_{2A}$R in post-mortem human PFC

The results arisen from the current study demonstrate the existence of constitutive 5-$HT_{2A}$R basal activity towards $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein subtypes in native human and mice brain cortex.

Up to now, the existence of constitutive activity of 5-$HT_{2A}$R represented an unresolved issue and a real pharmacological challenge ( Deurwaerdère et al., 2020). As mentioned before, constitutive activity is the ability of a receptor to be spontaneously active in absence of agonist occupancy (Kenakin, 1996; Milligan & Bond, 1997; Berg & Clarke, 2018). This property has been well characterized in heterologous expression systems *in vitro* (Costa & Herz, 1989; Kenakin, 1995; Milligan & Bond, 1997; Egan et al., 1998; Milligan et al., 1999; Berg et al., 2008). Nonetheless, in most cases, the receptor and/or Gα-protein expression levels exceeded the physiological GPCR expression values. Consequently whether constitutive activity observed at such enhanced expression levels is physiologically relevant remains unclear (Seifert & Wenzel-Seifert, 2002). To address this question, GPCR/Gα-protein stoichiometry should be kept at equivalent conditions to native tissues (Samama et al., 1993; MacEwan & Milligan, 1995; Seifert & Wenzel-Seifert, 2002; Aloyo et al., 2010).

The degree of constitutive receptor activity is considered as the reduction of basal effector activity produced by a full inverse agonist (Costa & Herz, 1989). Moreover, the decrease of constitutive activity must be sensitive to blockade with antagonist and be absent in knock-out animals for the involved receptor (Aloyo et al., 2009). Finally, the existence of basal receptor activity should be demonstrated in the absence of the potential influence of endogenous neurotransmitters (Seifert & Wenzel-Seifert, 2002). The presence of endogenous agonist constitutes a significant problem when analysing constitutive GPCR activity *in vivo* and in certain *in vitro* systems, such as neurons containing neurotransmitter vesicles. In this situation, discrimination

between endogenous agonists displacement by antagonists and reversion of basal constitutive activity by inverse agonists is crucial (Morisset et al., 2000; Wieland et al., 2001).

In this context, 5-$HT_{2A}$R displays the lowest ability among other 5-HT receptors to spontaneously activate intracellular signalling pathways when measuring the second messenger IP resulting of PLC activation (Berg et al., 2005; De Deurwaerdere et al., 2020). Attempts to demonstrate *in vitro* constitutive activity of this receptor have required receptor mutagenesis (Egan et al., 1998) or overexpression (Weiner et al., 2001), which is far from physiological circumstances.

The acquisition of the classical conditioning nictitating membrane eye blink response in rabbits represents an *in vivo* model of associative learning to demonstrate the presence of constitutively active 5-$HT_{2A}$R in brain (Welsh et al., 1998a; Harvey, 2003; Harvey et al., 2004; Romano et al., 2006). Several studies support that eye blink response is a response entirely mediated through 5-$HT_{2A}$R (Welsh et al., 1998a; Romano et al., 2000). Harvey's study revealed that agonist, like LSD, enhanced associative learning while some antagonist, like ketanserin, had no effect in learning, acting as neutral antagonist. Finally, MDL-11,939 and ritanserin where termed inverse agonist as they had the ability to retard learning. Constitutive activity of 5-$HT_{2A}$R has been confirmed by using 5,7 dihydroxytryptamine, a neurotoxin that produced depletion of the endogenous ligand 5-HT (Romano et al., 2006). It is obvious that proposed pharmacological properties of 5-$HT_{2A}$R drugs in this paradigm are discordant with the findings of the present *in vitro* study.

Previous *in vitro* studies have demonstrated, indirectly, the presence of constitutive 5-$HT_{2A}$R activity in human post-mortem PFC. Thus, it was shown that addition of the non-hydrolysable GTP analogue Gpp(NH)p, which uncouples receptor from Gα-proteins, increased the 5-$HT_{2A}$R population identified by the inverse agonist altanserin (Muguruza et al., 2013). The finding

was interpreted as the existence of a 5-$HT_{2A}$R population in Gα-protein precoupled conformation.

The fact that 5-$HT_{2A}$R can signal through multiple transducers (González-Maeso et al., 2003; Kurrasch-Orbaugh et al., 2003b; González-Maeso et al., 2007) should be considered when describing 5-$HT_{2A}$R constitutive activity because the coupling to the different Gα-proteins implies the existence of separate basal activities for each of them. However, most of the previous *in vitro* studies about 5-$HT_{2A}$R constitutive activity were limited to a single signalling pathway and were performed in cell cultures.

In this context, the present study performed [$^{35}$S]GTPγS SPA combined with immunodetection of Gα-protein subunits to evaluate the basal constitutive 5-$HT_{2A}$R coupling to the different Gα-proteins. Probably, this study provides the first direct evidence of constitutive 5-$HT_{2A}$R activity in human brain. Most of the previous works aiming to study constitutive activity are limited to measurement of second messengers (Berg et al., 1998), which represents an indirect, and likely unspecific, measurement of different Gα-protein activation. SPA technique allows directly measurement of each Gα-protein activity in response to receptor activation (Diez-Alarcia et al., 2021b). Moreover, this methodology measures functional consequence at the earliest receptor-mediated event avoiding the uncertainty of general responses by cross interactions in the next steps of signalling pathways (Harrison & Traynor, 2003).

Overall, the present study demonstrated that the coupling of 5-$HT_{2A}$R to Gα-proteins in human PFC shows constitutive activity. This is based on the fact that [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein can be inhibited by different 5-$HT_{2A}$R ligands with inverse agonist activity. In order to fulfil conditions to support the existence of constitutive activity, the decrease of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-protein in presence of altanserin, pimavanserin and volinanserin, and to $G\alpha_{q/11}$-protein in presence of volinanserin was blocked by selective 5-$HT_{2A}$R antagonists. Furthermore, the absence of activity in 5-

$HT_{2A}R^{(-/-)}$ mice confirmed the inverse agonist properties of the selective 5-$HT_{2A}R$ drugs chosen. Notably, the basal activities in 5-$HT_{2A}R^{(-/-)}$ and 5-$HT_{2A}R^{(+/+)}$ mice were very similar, indicating that 5-$HT_{2A}R$ precoupling to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins only represents a small fraction of the total basal activity of these Gα-proteins.

It should also be mentioned that under the present experimental conditions, the processing to obtain membrane-enriched fractions was based on strong centrifugation and washouts, which precludes the presence of significant 5-HT concentration in the medium. This condition excludes the possibility that the results obtained with inverse agonist candidate drugs could be due to the displacement of residual endogenous ligand. Even more, if that possibly was true and 5-HT was present, the drugs would be lack of Gα-protein subtype selectivity and all of them would behave as apparent inverse agonist.

In contras to 5-$HT_{2A}R$, it is well established the presence of constitutive activity of 5-$HT_{2C}R$ based on *in vitro* and *in vivo* data (Berg et al., 1999; Herrick-Davis et al., 2000; Rauser et al., 2001; Deurwaerdère et al., 2004). In fact, the critical role of the 5-$HT_{2C}R$ constitutive activity on the DA release in CNS has been elegantly demonstrated by *in vivo* microdialysis ( Deurwaerdère et al., 2004). Interestingly, the demonstration that 5-$HT_{2C}R$ inverse agonists present capacity to reduce basal IP accumulation but not AA release indicates differences in constitutive receptor activity across the different signal pathways (Berg at al., 1999). Therefore, it is mandatory to consider the different signal pathways when the study of potential receptor constitutive activity is planned.

## 5.3 5-$HT_{2A}$R functional coupling and constitutive activity in schizophrenia

The present study demonstrates a selective increase of constitutive activity of 5-$HT_{2A}$R on the $G\alpha_{i1}$-protein-mediated pathway in DLPFC of schizophrenia subjects. This signalling pathway is considered a pro-hallucinogenic route and the enhanced constitutive activity probably promotes a higher coupling efficiency of 5-$HT_{2A}$R in schizophrenia. In contrast, no changes were found in the 5-$HT_{2A}$R coupling to $G\alpha_{q/11}$-protein pathway.

In order to evaluate constitutive activity in brain tissue, previously well-characterized 5-$HT_{2A}$R inverse agonists were used. Among tested compounds, pimavanserin showed the highest inverse efficacy on 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins. However, pimavanserin was not suitable to evaluate 5-$HT_{2A}$R constitutive activity coupling to $G\alpha_{q/11}$-proteins because the compound shows neutral antagonist profile on this pathway. Therefore, in order to test both signalling pathways with the same compound, volinanserin was chosen as a suitable inverse agonist on 5-$HT_{2A}$R coupling to both $G\alpha_{q/11}$- and $G\alpha_{i1}$-proteins in post-mortem human brain of schizophrenia subjects. Volinanserin showed lesser inverse efficacy on $G\alpha_{i1}$-proteins than pimavanserin but was the only compound with 5-$HT_{2A}$R inverse agonist properties on $G\alpha_{q/11}$-proteins. The selectivity of pimavanserin and volinanserin was confirmed co-incubating with the 5-$HT_{2A}$R reference antagonist MDL-11,939. Therefore, pimavanserin and volinanserin were selected as suitable tools to further study 5-$HT_{2A}$R constitutive activity in PFC of subjects with schizophrenia and controls.

The present study compared basal constitutive activity of 5-$HT_{2A}$R coupling to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins among schizophrenia, non-schizophrenia suicide, and controls. Different concentrations of pimavanserin ($10^{-10}$ M, $10^{-8}$ M and $10^{-6}$ M) and volinanserin ($10^{-8}$ M, $10^{-6.5}$ M and $10^{-5}$ M) were chosen to delineate the maximal inhibitory effect ($I_{max}$) and the potency (-log$IC_{50}$) in conditions

being compatible with the availability of human tissue and economic resources. The respective concentrations were selected based on the concentration-response curves of the previously performed inverse agonist characterization. A non-schizophrenia suicide group was introduced to test the selectivity of findings in schizophrenia and to control for the potential confounding effect of suicide as death mechanism in certain cases of schizophrenia.

The inhibitory effect of pimavanserin and volinanserin on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins was higher in schizophrenia (20% and 15% inhibition, respectively) compared with non-schizophrenia suicide (14% and 14% inhibition, respectively) and control subjects (14% and 13% inhibition, respectively). In contrast, the inhibition produced by volinanserin on the [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was similar between schizophrenia (15% inhibition), non-schizophrenia suicide (16% inhibition) and control (15% inhibition) subjects.

The findings are in agreement with Garcia-Bea et al. who showed a significant enhanced 5-$HT_{2A}$R-mediated $G\alpha_{i1}$-protein activation by the 5$HT_{2A}$R agonist (±)DOI in post-mortem DLPFC of schizophrenia subjects. The study also demonstrated unaltered 5-$HT_{2A}$R-mediated $G\alpha_{q/11}$-protein activation by (±)DOI in the same subjects and tissue preparation. As (±)DOI is an agonist, these results revealed a supersensitivity of 5-$HT_{2A}$R coupling to the $G\alpha_{i1}$-protein pathway. However, whether these findings represented enhanced constitutive activity needed further demonstration.

In the present study, and to confirm the existence of (±)DOI-induced supersensitive coupling to the $G\alpha_{i1}$-protein pathway, the effect of (±)DOI was also evaluated in the same subject groups where inverse agonists have been tested. This positive control should reproduce previous results (García-Bea et al., 2019) and would contribute to support the hypothesis of enhanced constitutive activity of 5-$HT_{2A}$R in schizophrenia. Since (±)DOI is a 5-$HT_{2A/2C}$R

agonist, the selectivity of the obtained results was confirmed by co-incubation with the selective 5-$HT_{2A}$R antagonist MDL-11,939. Thus, stimulatory effects induced by (±)DOI on [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins were also analysed at a single concentration ($10^{-5}$ M / 10 μM) that induces submaximal effect. As expected, (±)DOI-induced stimulatory effect on $G\alpha_{i1}$-protein activity was enhanced in schizophrenia (15% stimulation) compared to non-schizophrenia suicide (9% stimulation) and control (10% stimulation) subjects. The enhanced (±)DOI effects in schizophrenia were not observed in the 5-$HT_{2A}$R-mediated activation of the $G\alpha_{q/11}$-protein pathway. In the same way, a recent study (Odagaki et al., 2021) concluded no differences in the functional coupling of 5-$HT_{2A}$R to the canonical $G\alpha_{q/11}$-protein pathway in PFC of subjects with schizophrenia.

The correlation analyses between the maximal inhibitory effects of pimavanserin, volinanserin and (±)DOI showed statistical positive relationships for $G\alpha_{i1}$-protein activation. This result reinforces the hypothesis that the three compounds identify similar functional 5-$HT_{2A}$R conformations. However, no statistical significant correlation was seen for $G\alpha_{q/11}$-proteins when maximal effect of (±)DOI and inhibitory effect of volinanserin were correlated. These results could be reflecting a differential interaction of 5-$HT_{2A}$R with $G\alpha_{q/11}$-proteins in function of the ligand and/or perhaps partial efficacy properties of the drugs (Brea et al., 2009).

Altogether, these findings and previous radioligand binding studies (González-Maeso et al., 2008; Muguruza et al., 2013; Diez-Alarcia et al., 2021a) confirm the hypothesis of an enhanced proportion of agonist-sensitive 5-$HT_{2A}$R in post-mortem DLPFC of schizophrenia subjects. Aforementioned binding studies suggested an alteration in the rate of conformational receptor states towards the active conformation leading to increased functional coupling rather than alterations in total 5-$HT_{2A}$R density (Diez-Alarcia et al., 2021a). Moreover, 5-$HT_{2A}$R expression in the same brain area was not altered in schizophrenia

subjects, neither when evaluated using immunodetection or HTR2A mRNA expression (Garcia-Bea et al., 2019; Zhao et al., 2022).

It should take into account that basal Gα-protein activity is the consequence of stoichiometric coupling between receptor, Gα-protein and, probably, other protein complexes. Therefore, not only alterations of 5-$HT_{2A}$R expression should be explored as contributors to the enhanced receptor coupling and constitutive activity. Modified Gα-protein expression might be another factor influencing the stoichiometric coupling and the subsequent functional responses. In order to discard this possibility in brain of schizophrenia subjects, Western blot experiments were performed to evaluate $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein immunoreactivity in cortical membranes of all subjects. Results confirmed no changes in the immunoreactivity of either Gα-protein. Thus, enhanced constitutive activity seems not to be related to an altered expression of Gα-proteins that could influence the functional coupling to 5-$HT_{2A}$R (Garcia-Bea et al., 2019).

Spite of the little evidence showing enhanced 5-$HT_{2A}$R constitutive activity in schizophrenia subjects, some studies support a central serotonergic tone alteration in the disorder. Measurement of prolactin response to the 5-HT-releasing drug d-flenfluramine is considered an *in vivo* test dependent on 5-$HT_{2A/2C}$R activation, and this test showed an enhanced activation in drug-free schizophrenia subjects (Abel et al.,1996; Jones et al., 1998; Monteleone et al., 1999).

Basal [$^{35}$S]GTPγS binding to $G\alpha_{i1}$- and $G\alpha_{q/11}$-protein were not different between schizophrenia, non-schizophrenia suicide and control subjects. The lack of changes in basal [$^{35}$S]GTPγS binding does not represent an argument in favour of unaltered constitutive activity in schizophrenia. The detection of altered basal [$^{35}$S]GTPγS binding coupling to a single GPCR is difficult because it represents the total signalling of all Gα-proteins activity. Therefore, signal / noise detection of a GPCR overactive coupling might be technically

complicated. As previously discussed, in 5-$HT_{2A}R^{(-/-)}$ mice basal [$^{35}$S]GTPγS binding values to $G\alpha_{i1}$- and $G\alpha_{q/11}$-proteins were not different to those obtained in 5-$HT_{2A}R^{(+/+)}$ mice, confirming that unaltered or altered 5-$HT_{2A}$R constitutive coupling to these proteins represents a low rate of the respective total basal Gα-protein activity.

In the context of schizophrenia research, studies in human tissue, and especially in post-mortem brain studies, provide valuable information about molecular alterations underlying the neurobiology of the disease. However, these studies possess their own inherent difficulties, including high interindividual variability comparing to animal models. Therefore, the possible confounding effect of demographic and clinical measures, such as, sex, age, PMD and storage time should be considered (McCullumsmith et al., 2014). In the present work, subjects were individually matched for these variables, and significant differences for confounding factors between groups were absent. The only factor that was different between groups corresponded to storage time of samples at -80ºC until assays. Therefore, it was included as covariate factor in the statistical analysis. None of the analyses was altered when storage time was considered as confounding factor. Furthermore, 5-$HT_{2A}$R expression has classically demonstrated a negative correlation with age (González-Maeso et al., 2008; Muguruza et al., 2013; Diez-Alarcia et al., 2021a). Therefore, and although cases and controls were relatively matched for the age, it was considered together with sex, PMD and storage time as covariate factor when studying the expression or function of 5-$HT_{2A}$R.

Chronicity of the disease and antipsychotic treatment are two factors that may confound biological observations in tissue of affected subjects. In this sense, studies with treatment-naïve patients are considered valuable to test whether alterations in 5-$HT_{2A}$R are present independently of pharmacological treatment. This possibility is feasible in first-episode psychosis patients and under *in vivo* conditions. However, post-mortem studies in subjects with

schizophrenia are always performed on samples of subjects that received antipsychotic prescription once the diagnosis was established. This fact is complementary to the low compliance with pharmacological treatments in schizophrenia patients (McCullumsmith et al., 2014). The schizophrenia subjects included in the present study were considered antipsychotic free, due to the absence of antipsychotic drugs in blood toxicological screening at the time of autopsy. Of course, lack of antipsychotics drugs detected in blood at autopsy does not imply absence of treatment at all. Moreover, to confirm the absence of antipsychotics further analyses were performed in brain tissue. Brain toxicology confirmed in all subjects the absence of active toxicological concentrations of psychotropic drugs that could be affecting the functional study (Musshoff et al., 2020). Second generation antipsychotics present high affinity for 5-$HT_{2A}$R (Richelson & Souder, 2000) and could modulate 5-$HT_{2A}$R expression and/or function, after chronic treatment (Grey & Roth, 2000; González-Maeso et al., 2008; Garcia-Bea et al., 2019). On the other hand, the residual presence of drugs with affinity for 5-$HT_{2A}$R could block the binding of pimavanserin, volinanserin and (±)DOI to the receptor (Dean et al., 2008). In both possibilities, 5-$HT_{2A}$R functionality would be apparently decreased in schizophrenia, an opposite result to findings of the present study.

Whether chronic antipsychotic treatment down-regulates 5-$HT_{2A}$R expression and the contribution of this modulation to their therapeutic effect is still a matter of debate. Several studies support that schizophrenia subjects treated with antipsychotics show a reversion of the enhanced HTR2A mRNA expression and protein immunoreacivity observed in PFC of antipsychotic-free schizophrenia subjects (Burnet et al., 1996; Hernandez & Sokolov, 2000; López-Figueroa et al., 2004; Garcia-Bea et al., 2019; Zhao et al., 2022). Radioligand binding assays in post-mortem brain also showed a reversion of the enhanced 5-$HT_{2A}$R density when schizophrenia subjects have antipsychotic in blood (González-Maeso et al., 2008; Muguruza et al., 2013;

Diez-Alarcia et al., 2021a). The finding is similar to observations in rodent brain where second generation antipsychotics induce changes in 5-$HT_{2A}$R through modulation of their expression (González-Maeso et al., 2008; Yadav et al., 2011; Kurita et al., 2012; García-Bea et al., 2019). Blockade of the radioligand binding site due to residual presence of antipsychotics acting as 5-$HT_{2A}$R antagonists (Dean et al., 2008; Garcia-Bea et al., 2019) is also an alternative explanation for this down-regulation. Therefore, it is tempting to speculate that chronic antipsychotic treatment down-regulates 5-$HT_{2A}$R expression to counterbalance the receptor supersensitivity due to enhanced constitutive 5-$HT_{2A}$R. However, currently no information about the 5-$HT_{2A}$R coupling to the different Gα-proteins following chronic antipsychotic treatment is available either in normal or schizophrenia-like animal models. The present study strongly suggests that in order to discard eventual influences of antipsychotics in post-mortem studies, independent groups of antipsychotic-free and antipsychotic-treated subjects should be selected and independently analysed.

It is worth mentioning that 13 subjects out of 23 schizophrenia subjects had died by suicide. Due to the existence of this confounding factor, that is not present in control subjects, a group of suicide victims with other psychiatric disorders than schizophrenia was included. These subjects were diagnosed of personality disorder, anxiety and obsessive-compulsive disorder. It is well known that mortality risk for suicide is increased in subjects with psychiatric disorders, including schizophrenia (Bachmann, 2018). Moreover, numerous abnormalities have been reported in the serotonergic system in suicide victims (Stockmeier et al., 1997). The results of the present study suggest that the enhanced constitutive activity of 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins is likely related to schizophrenia status, rather than suicidal behaviour in the disease process. The findings are in concordance with Odagaki et al., who reported no alterations in the functional coupling of brain 5-$HT_{2A}$R to $G\alpha_{q}$-proteins in

different psychiatric disorders. Previous 5-$HT_{2A}$R binding studies in DLPFC also described no changes in non-schizophrenia suicide subjects (Muguruza et al., 2013).

The enhanced constitutive activity of 5-$HT_{2A}$R inhibited by pimavanserin and volinanserin in schizophrenia involves the $G\alpha_{i1}$- but not $G\alpha_{q/11}$-protein signalling. This fact indicates that the functional alteration observed is selective for the hallucinogenic signal transduction pathway of 5-$HT_{2A}$R, i.e., the activated by LSD, psylocibin, DOB, (±)DOI and other hallucinogenic agonist drugs. Whether other non-canonical Gα-protein and Gα-protein independent pathways are altered in schizophrenia remains to be studied. As mentioned, 5-$HT_{2A}$R couple with a wide variety of intracellular signalling pathways and activates multiple intracellular mechanisms (Roth, 2011). In this context, and according to the current literature data, the status of the β-arrestin-dependent coupling of 5-$HT_{2A}$R in brain of subjects with schizophrenia represents a priority of maximum interest. However, for this purpose, technical challenges must be overcome (Diez-Alarcia et al., 2021b; Pottie & Stove, 2022).

## 5.4 5-$HT_{2A}$R biased and inverse agonism properties of antipsychotic drugs

Currently, most of the approved drugs to treat schizophrenia share affinity for $D_2$R and 5-$HT_{2A}$R. However, antipsychotics only ameliorate the positive symptoms of schizophrenia and some patients are resistant to conventional treatment (Legge et al., 2020). Nowadays, dopaminergic antagonism seems to be essential for antipsychotic activity (Miyamoto et al., 2005). Nevertheless, this antagonism is highly related with collateral adverse effects, like extrapyramidal symptoms and hyperprolactinemia (Leucht et al., 2013). By contrast, atypical antipsychotics display lower affinity for $D_2$R than for $5HT_{2A}$Rs, which improves the side-effects profile (Meltzer et al., 1989). Based on this evidence, compounds with highly selective 5-$HT_{2A}$R antagonist profile and no dopaminergic implication were developed and tested for the treatment of schizophrenia (Richelson & Souder, 2000; Jones et al., 2020).

Once the enhanced constitutive activity of 5-$HT_{2A}$R through the pro-hallucinogenic pathway in DLPFC of schizophrenia subjects was shown, inverse agonism should be considered as potential antipsychotic therapeutic strategy. Neutral antagonists compete for the same orthosteric binding site, preventing the cellular response induced by exogenous or endogenous agonists, and showing no effect in the constitutive activity. In contrast, inverse agonists, which preferentially bind and stabilize the GPCR in an inactive state (Kenakin, 2002), should be more useful than neutral antagonists for the inactivation of constitutively active receptors (Weiner et al., 2001). In fact, many drugs previously thought to be competitive antagonists actually show intrinsic activity as inverse agonist at different monoaminergic receptors (Egan et al., 1998; Sullivan et al., 2015).

Among all the drugs evaluated in the present study, altanserin, pimavanserin and volinanserin displayed selective inverse efficacy in the 5-$HT_{2A}$R coupling

to $G\alpha_{i1}$-proteins and, therefore, could be a good starting point for the development of new antipsychotic drugs.

Volinanserin is the first drug showing high selectivity on 5-$HT_{2A}$R that was tested for schizophrenia in a clinical trial (Paulis, 2001). Firstly, it showed higher efficacy than placebo but less than haloperidol, and the compound was discontinued (Paulis, 2001). Even if the development of volinanserin was discontinued, this drug is still widely used as selective pharmacological tool for quantification and visualization of 5-$HT_{2A}$R.

Pimavanserin has gained FDA approval to reduce delusions and hallucinations in Parkinson´s disease (Cummings et al., 2014), being the first approved antipsychotic lacking dopaminergic affinity (Hacksell et al., 2014). Recently, pimavanserin has shown to reduce negative symptoms in schizophrenia patients (Bugarski-Kirola et al., 2022). Until now, pimavanserin has been studied as adjunctive therapy for schizophrenia, with lesser side effects than risperidone and haloperidol (Meltzer et al., 2012).

There is no literature evidence that altanserin, considered an analogous of ketanserin, has been tested for the treatment of schizophrenia.

Overall, it can be drawn that antipsychotic-mediated clinical effect can be obtained by 5-$HT_{2A}$R antagonism / inverse agonism (Jones et al., 2020; Abbas & Roth, 2008). However, the clinical effects of pimavanserin and volinanserin appear to be not sufficient for the treatment of schizophrenia. Unfortunately, there is not data describing clinical use of pimavanserin in monotherapy for schizophrenia and the comparison vs conventional antipsychotics. According to present results, combination clinical studies of selective 5-$HT_{2A}$R inverse agonists, as pimavanserin, with an antipsychotic that possesses 5-$HT_{2A}$R antagonist properties would probably result in lack of beneficial effects, as both ligands compete to bind the same receptor. Moreover, it has been described a serotonergic subtype of schizophrenic patients, showing more pronounced

disturbances on serotonergic system, that could benefit using potent and selective 5-$HT_{2A}$R inverse agonists (Baltzersen et al., 2020). Thus, further research is needed to identify the clinical impact of these novel drugs in schizophrenia pharmacotherapy.

Most of the atypical antipsychotics currently in clinical use are described, among their multiple pharmacological profile, as 5-$HT_{2A}$R antagonists (Meltzer et al., 1989; Meltzer et al., 2003; Meltzer & Huang, 2008). However, it is being progressively recognized that some of the clinically effective antipsychotic drugs are, in fact, 5-$HT_{2A}$R inverse agonist (Egan et al., 1998; Winer et al., 2001; Aloyo et al., 2009; Fribourg et al., 2011; Sullivan et al., 2015). The pharmacological characterization of inverse agonism profile for antipsychotics has been mainly developed in heterologous systems, while inverse agonism studies in physiological conditions are scare. Indeed, mutagenic studies of 5-$HT_{2A}$R have revealed that this receptor displays constitutive activity that helps to unmask the partial inverse agonism of different antipsychotics (Egan et al., 1998). Moreover, there are few reports studying the functional selective and biased signalling profile of antipsychotics, beyond dopamine receptors (Komatsu et al., 2019; Von Moo et al., 2022). Here, different signalling pathways of the 5-$HT_{2A}$R were considered for the characterization of the functional selectivity profile of antipsychotics, and the evaluation was not only limited to the canonical $G\alpha_{q/11}$-protein pathway.

Among the tested antipsychotic drugs, clozapine, olanzapine and risperidone showed inverse agonist profile on the pro-hallucinogenic $G\alpha_{i1}$-protein-mediated 5-$HT_{2A}$R signalling pathway. Paliperidone also displayed inverse agonist profile on $G\alpha_{i1}$- and $G\alpha_{i2}$-proteins. The antagonist MDL-11,939 was able to block completely the inhibitory effects on $G\alpha_{i2}$-protein signalling pathway while $G\alpha_{i1}$-protein effect of paliperidone was only partially blocked. These results rise the relevancy of inverse agonist effect on the 5-$HT_{2A}$R

coupling to $G\alpha_{i1}$-proteins as mechanism to explain antipsychotic efficacy and to develop new antipsychotics.

In contrast, inverse agonist pharmacological properties on the 5-$HT_{2A}$R coupling to $G\alpha_{q/11}$-proteins were not found for any of the antipsychotic tested. Inhibitory effect of risperidone on basal [$^{35}$S]GTPγS binding was not sensitive to the selective antagonist MDL-11,939, suggesting alternative effects on another GPCR.

The classical identification studies of the molecular profile of antipsychotics drugs utilize ligand-binding competition and single functional assays as methodological screening approaches. Although it is usual to find complete maps and tables of antipsychotic affinities for a wide range of receptors, equivalent data for multiple signalling pathways are scarce. Even more, evaluation of multiple cellular pathways as consequence of interaction between each receptor and specific antipsychotic drug is almost absent. Native tissues have revealed a tremendous degree of heterogeneity in potential responses to antipsychotic-receptor binding interactions, and have highlighted the lack of selectivity of most antipsychotics used in routine clinical (Weiner et al., 2001; Roth et al., 2004). Furthermore, the existence of functional selectivity and biased signalling adds complexity to the mechanisms involved in the therapeutic activity of antipsychotic drugs. In these sense, elucidation of functional selectivity and the clinical relevance of inverse agonism versus neutral antagonist are recognized among the most relevant current challenges in pharmacology (Berg & Clarke, 2018).

The present study reports that different antipsychotics with different affinities for aminergic receptors are able to induce bias signalling towards different Gα-protein subunits. In order to study the involvement of 5-$HT_{2A}$R, all drugs were tested in absence and in presence of the 5-$HT_{2A}$R reference antagonist MDL-11,939.

The typical antipsychotic haloperidol, that has been described as highly selective $D_2R$ antagonist (Janssen, 1967) only decreased the [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-protein and this effect was not blocked by MDL-11,939. Therefore, this inverse agonist profile of haloperidol at $G\alpha_{i1}$-protein is mediated via another GPCR that was not explored. Previous studies in cell culture demonstrated inverse agonist profile of haloperidol on $D_2R$ (Roberts & Stange, 2005; Strange, 2008)

Aripiprazole is classified as third generation antipsychotic with a unique pharmacological profile. This includes partial agonist at several GPCRs, like $D_2R$ and 5-$HT_{1A}R$, as well as lower antagonist actions at others like 5-$HT_{2A}R$ (Davies et al., 2004). Here, aripiprazole behaved as agonist for $G\alpha_o$-proteins with no effects on $G\alpha_{i1}$-, $G\alpha_{i2}$- and $G\alpha_{q/11}$-proteins. The stimulatory effects were not mediated by 5-$HT_{2A}R$ and could represent agonism on $D_2R$ and/or 5-$HT_{1A}R$, which are receptors coupled to inhibitory Gα-proteins (Strange, 2008).

In the same way, quetiapine also showed agonist properties on $G\alpha_{i1}$-proteins, with lack of response to 5-$HT_{2A}R$ antagonism. Quetiapine has previously been described as 5-$HT_{1A}R$ agonist, and this could be responsible of some of the effects observed (Han et al., 2019). Quetiapine also displays inverse agonist properties on $D_2R$ (Akam & Strange, 2004; Roberts & Strange, 2005) that were not explored in the present study.

The atypical antipsychotic olanzapine has been described as a 5-$HT_{2A}R$ antagonist with higher affinity for this receptor compared to $D_2R$ (Jafari et al., 2012). As previously mentioned, olanzapine inhibited [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-protein, being sensitive to 5-$HT_{2A}R$ antagonist, which confirms the role of 5-$HT_{2A}R$ in this inverse agonist effect.

Risperidone is a potent atypical antipsychotic with proposed antagonist affinity at serotonergic, dopaminergic, adrenergic and histaminergic receptors, and lacking of affinity for cholinergic muscarinic receptors (Cohen, 1994).

Risperidone exhibited inhibitory responses on all studied Gα-proteins but only the $G\alpha_{i1}$- and $G\alpha_{o}$-proteins were related to 5-$HT_{2A}$R inverse agonist profile. The coupling of 5-$HT_{2A}$R to $G\alpha_{o}$-proteins unmasked by risperidone inhibition represented a singular finding. In the first part of this Thesis, nelotanserin, volinanserin and, especially ritanserin demonstrated inhibitory effect on [$^{35}$S]GTPγS binding to $G\alpha_{o}$-proteins. However, the involvement of 5-$HT_{2A}$R in the actions of these drugs were not tested. Therefore, inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{o}$-proteins by risperidone informs the existence of 5-$HT_{2A}$R constitutive activity on this pathway. The antagonist effect exerted by SB 242084 on $G\alpha_{i1}$-protein inhibition by risperidone could be related to its affinity profile for 5-$HT_{2C}$R but also 5-$HT_{2A}$R are blocked when this compound is used in micromolar concentration (Kennet et al., 1997). Therefore, risperidone inhibition of [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins probably represents a 5-$HT_{2A}$R-related effect because is sensitive to the non-selective 5-$HT_{2A/C}$R antagonist ketanserin, the selective 5-$HT_{2A}$R antagonist MDL-11,939 and high concentrations of the partially selective 5-$HT_{2C}$R antagonist SB 242084. The implication of risperidone on $G\alpha_{q/11}$-protein inhibition was shown to be $\alpha_1$-adrenoceptor-mediated response, being sensitive to the α-adrenoceptor antagonist phentolamine and to ketanserin, a 5-$HT_{2A/2C}$R drug with moderate affinity for $\alpha_{1B}$-adrenoceptors (Israilova et al., 2002; Sathi et al., 2008)

The active metabolite of risperidone, paliperidone, showed a different functional selectivity profile that could explain differences observed between risperidone and paliperidone effects in cell culture assays (Clarke et al., 2013, Corena-McLeod, 2015). As previously mentioned, paliperidone displayed inverse agonist profile on $G\alpha_{i1}$-proteins with probably involvement of 5-$HT_{2A}$R and other GPCRs whereas inverse agonism on $G\alpha_{i2}$-proteins was fully sensitive to the antagonist MDL-11,939. Therefore, paliperidone displayed differential profile to its pro-drug risperidone with inverse agonist effect on $G\alpha_{i1}$- and $G\alpha_{i2}$-proteins and null effect on $G\alpha_{o}$- and $G\alpha_{q/11}$-proteins.

Finally, clozapine promoted inhibition of $G\alpha_{i1}$-proteins that was blocked by MDL-11,939, confirming the implication of 5-$HT_{2A}$R. In contrast, clozapine behaved as agonist on $G\alpha_{o}$- and $G\alpha_{q/11}$-protein activation, but these effects were not mediated via 5-$HT_{2A}$R. In addition to serotonin and dopamine receptors, clozapine also possesses affinity for α-adrenoceptors, cholinergic muscarinic and histaminergic receptors (Nucifora et al., 2017). This polypharmacological profile was explored by using selective antagonist drugs. Thus, the stimulatory effects of clozapine on $G\alpha_{q/11}$-protein were blocked by the muscarinic and $H_1$R antagonists atropine and cetirizine, respectively. Since there is no information about crossed affinity of cetirizine for muscarinic receptor subtypes and atropine for $H_1$Rs (Orzechowski et al., 2005), it is suggested that both GPCR might be implicated in the stimulation of clozapine on $G\alpha_{q/11}$-proteins.

Therefore, this study demonstrated a differential targeting of Gα-protein signalling modulation by antipsychotic drugs, compatible with the existence of functional selectivity in their activity on 5-$HT_{2A}$Rs. The results strength the relevancy of inverse agonism as the possible mechanism to reverse the enhanced 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-protein observed in schizophrenia. However, inverse agonist properties on 5-$HT_{2A}$R or other GPCRs is not a common pharmacological profile of all antipsychotics. The differential intrinsic activity and functional selectivity profile could explain the different clinical and side effect profile of antipsychotics. Overall, the present results shed light to description of functional selectivity and intrinsic activity of antipsychotics in post-mortem human brain. Lastly, the creation of a transgenic mouse model that exhibits increased 5-$HT_{2A}$R activity towards the pro-hallucinogenic $G\alpha_{i1}$-protein pathway might provide an excellent preclinical model to test antipsychotic activity.

# Conclusions

The main conclusions derived from this Doctoral Thesis are:

1. The 5-$HT_{2A}$R expressed in human PFC displays functional coupling to $G\alpha_{q/11}$-proteins but also with other Gα-proteins such as inhibitory $G\alpha_{i/o}$-proteins. Differential targeting profile of Gα-proteins is obtained after interaction of 5-$HT_{2A}$R with selective agonist, antagonist and inverse agonist drugs. It is concluded that functional selectivity is present for 5-$HT_{2A}$R coupling to Gα-proteins in human PFC.

2. The functional coupling of 5-$HT_{2A}$R to $G\alpha_{q/11}$- and $G\alpha_{i1}$-proteins in human PFC shows constitutive activity. Different drugs previously described as neutral antagonist were able to decrease the basal [$^{35}$S]GTPγS binding to Gα-proteins, suggesting inverse agonist properties. The blockade of the inhibitory effect in presence of 5-$HT_{2A}$R antagonists and the absence of this pharmacological feature in 5-$HT_{2A}R^{(-/-)}$ knock-out mice allowed us to identify the compounds as 5-$HT_{2A}$R inverse agonist.

3. Antibody-capture [$^{35}$S]GTPγS scintillation proximity assay (SPA) is a suitable method for the evaluation of constitutive activity and functional selectivity of GPCRs in native tissue. This possibility opens new perspectives for study of human brain disorders involving GPCR alterations.

4. Altanserin and pimavanserin displayed preferential inverse agonist effects on 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins, with no effect on $G\alpha_{q/11}$-proteins. Volinanserin showed unbiased 5-$HT_{2A}$R inverse agonism on $G\alpha_{q/11}$- and $G\alpha_{i1}$-proteins. Ketanserin revealed a biased agonist/antagonist profile on 5-$HT_{2A}$R coupling, behaving a partial agonist on $G\alpha_{q/11}$-proteins and lacking of effect on $G\alpha_{i1}$-proteins. MDL-11,939 was the only drug that displayed neutral

antagonism activity on all Gα-proteins. Finally, nelotanserin, ritanserin and eplivanserin showed different signaling profiles that were not exclusively dependent on 5-$HT_{2A}$R interaction.

5. The inhibition induced by pimavanserin and volinanserin on the [$^{35}$S]GTPγS binding to $G\alpha_{i1}$-proteins was higher in PFC of subjects schizophrenia compared to non-schizophrenia suicide and control groups. In contrast, the inhibitory effect exerted by volinanserin on the [$^{35}$S]GTPγS binding to $G\alpha_{q/11}$-proteins was similar between groups. It is concluded that a selective increased basal constitutive activity of 5-$HT_{2A}$R coupling to the $G\alpha_{i1}$-protein-mediated pro-hallucinogenic pathway is present in schizophrenia subjects free of antipsychotic treatment.

6. The 5-$HT_{2A}$R agonist (±)DOI displayed enhanced stimulatory effect on the 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins in schizophrenia subjects compared to non-schizophrenia suicide and control groups. However, no changes were observed in the stimulation induced by (±)DOI on 5-$HT_{2A}$R coupling to $G\alpha_{q/11}$-proteins. The results are compatible with the existence of elevated constitutive 5-$HT_{2A}$R activity in brain of subjects with schizophrenia.

7. Antipsychotic drugs displayed functional selectivity on the different Gα-proteins in human post-mortem PFC. Differences exist according to the pharmacological profile of the respective drug. Inverse agonist properties on 5-$HT_{2A}$R coupling to $G\alpha_{i1}$-proteins was a common profile of clozapine, olanzapine, risperidone and paliperidone. No inverse agonism on 5-$HT_{2A}$R coupling to $G\alpha_{q/11}$-proteins was observed. Therefore, the enhanced $G\alpha_{i1}$-protein-mediated constitutive activity of 5-$HT_{2A}$R in schizophrenia represents

a pharmacological target for current and future inverse agonist antipsychotic drugs.

8. Together with functional selectivity on 5-$HT_{2A}$R coupling, antipsychotic exhibited a multiple pharmacological profile on [$^{35}$S]GTPγS binding to diverse Gα-proteins in human PFC Clozapine, in addition to 5-$HT_{2A}$R activity, stimulated $G\alpha_{q/11}$-proteins through cholinergic muscarinic and/or histamine $H_1$ receptors. Risperidone inhibited $G\alpha_{q/11}$-proteins via $\alpha_1$-adrenoceptors and showed inverse agonist 5-$HT_{2A}$R activity on $G\alpha_{i1}$- and $G\alpha_o$-proteins. Quetiapine, haloperidol and aripiprazole induced a variety of functional responses on Gα-protein activation that were mediated by a GPCR other than 5-$HT_{2A}$R

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# 5-$HT_{2A}$ hartzailearen aktibitate konstitutiboa eta farmakoen hautakortasun funtzionala: eskizofrenikoen post-mortem garunean egindako lana


**Itziar Muneta Arrate**

## LABURDUREN ZERRENDA

| | |
|---|---|
| [$^{35}$S]GTPγS | 35 sufrearekin markaturik dagen guanosina-5´-O-(γ-tio)-trifosfatoa |
| 5-HT | 5-Hidroxitriptamina (serotonina) |
| 5-$HT_{2A}$R | Serotonina 2A hartzailea |
| 5-$HT_{2A}R^{(-/-)}$ | *Knock-out* |
| 5-$HT_{2A}R^{(+/+)}$ | *Wild-Type* |
| 7TM | 7 domeinuko mintz zeharreko hartzailea |
| AA | Azido arakidonikoa |
| AC | Adenilato ziklasa |
| BB | Finkapen basala |
| Bolinanserin | MDL100907 |
| BRET | Bioluminezentzia-erresonantzia bidezko energia-transferentzia |
| $Ca^{2+}$ | Kaltzio ioia |
| CaMKII | Kaltzio/kalmodulinaren mendeko kinasa II |
| cAMP | Adenosina monofosfato ziklikoa |
| $D_2$R | Dopamina 2 hartzailea |
| DA | Dopamina |
| DAG | Diazilglizerola |
| DLPFC | Aurre-garunazal dortsolaterala |
| (±)DOI | 2,5-dimetoxi-4-iodoamfetamina |
| DSM | Gaixotasun mentalen diagnosia eta estatistika eskuliburua |
| $EC_{50}$ | Estimulazio efektu maximoa lortzeko % 50 kontzentrazioa |
| $E_{max}$ | Estimulazio efektu maximoa |
| ERK | Zelulaz kanpoko seinaleek erregulaturiko kinasak |
| FDA | Drogen eta elikagaien administrazioak |
| GABA | Azido-aminobutirikoa |

| | |
|---|---|
| GPCR | G-proteinei loturiko hartzaileak |
| GDP | Guanosina difosfatoa |
| GRK | G-proteinei lotutako kinasa |
| GTP | Guanosine trifosfatoa |
| GTPγS | 5'-O-[gamma-tio]trifosfatoa |
| GWAS | Genoma-osoaren asoziazio-azterketa |
| $IC_{50}$ | Inhibizio efektu maximoa lortzeko % 50 kontzentrazioa |
| $I_{max}$ | Inhibizio efektu maximoa |
| $IP_3$ | Inositol 1,4,5-trifosfatoa |
| LSD | Azido lisergikoaren D-dietilamida |
| MAPK | Aktibatutako mitogenoen proteina kinasa |
| NBS | Finkapen ez espezifikoa |
| NMDA | N-Metil-D-aspartatoa |
| NSZ | Nerbio sistema zentrala |
| PCP | Fentziklidina |
| PET | Positroien igorpen bidezko tomografia |
| $PIP_2$ | Fosfatidilinositol 4,5-bifosfatoa |
| PKC | Proteina kinasa C |
| $PLA_2$ | $A_2$ fosfolipasa |
| PLC | C fosfolipasa |
| PMD | Post-mortem atzerapena |
| PSD-95 | 95 dentsitate proteina postsinaptikoa |
| PTX | *Bordetella pertussis* toxina |
| RSK-2 | S6 kinasa erribosomala |
| SNP | nukleotido bakarreko polimorfismoa |
| SPA | Immunoprezipitazioarekin akoplatutako $[^{35}S]$GTPγS finkapen teknika |

# Sarrera

## 1.1 Eskizofrenia

### 1.1.1 Definizioa

Eskizofrenia ezgaitasun soziala eragiten duen gaixotasun kronikoa da. Haluzinazioak, anhedonia eta nahasmendu kognitiboak eskizofrenia gaixotasunaren ezaugarri nabarmenak dira (Owen et al., 2016; Jauhar et al., 2022). Munduko biztanleriaren %0,3 eta %0,7ak eskizofrenia gaixotasuna pairatzen duela kalkulatzen da (McGrath et al., 2008), etaeskizofreniaren garapenak maiztasun handiagoa du gizonetan emakumetan baino (Jongsma et al., 2019). Orokorrean, lehenengo gertakari psikotikoa nerabezaro berantiarrean edo helduaro goiztiarrean garatzen da. Gizonetan gaixotasunaren hasiera nerabezaroan eman ohi da (Kirbride et al., 2012); emakumeetan, adiz, hogeiko hamarkadaren amaieratik hogeita hamarreko hamarkadaren hasiera artean (Ochoa et al., 2012).

Eskizofrenia bizi itxaropena 10-20 urte artean laburtu dezakeen gaixotasun psikiatriko larrienetarikoa da (Chesney et al., 2014). Bestalde, suizidioa heriotza kausa arrunta da paziente eskizofrenikoen artean, %5eko bizi arriskua suposatzen duena (Hor eta Taylor, 2010).

### 1.1.2 Sintomatologia

Eskizofreniaren diagnosia historia klinikoan eta egoera mentalaren azterketan oinarrituz egiten da. Gaur egun, ez dago diagnosirako frogarik , ezta biomarkadorerik eskuragarri, beraz, eskizofreniaren diagnostikoa sintomatologia klinikoan oinarritzen da. Horretarako, Estatu Batuetako Psikiatriako Elkartearen “Gaixotasun mentalen diagnosia eta estatistika eskuliburua” (DSM), edo Munduko Osasun Erakundearen “gaixotasunen sailkapen estatistiko internazionala” (CIE) erabiltzen dira (Kendler, 2016).

Eskizofrenia psikopatologia anitzeko gaixotasuna da, non, adierazpen klinikoak hiru kategoria nagusitan biltzen diren: sintoma positiboak, negatiboak eta kognitiboak. Sintoma positiboen artean haluzinazioak (entzumen motakoak maiztasun handiagoarekin), ameskeriak eta portaera psikotikoak azaltzen dira maiztasun handiagorekin. Sintoma positiboak gertaeraka agertu ohi dira, eta, oro har, denbora-tarteetan, nahiz eta gaixo batzuek eguneroko-sintoma psikotikoak izaten dituzten epe luzera. Sintoma negatiboen ezaugarri nagusiak anhedonia, apatia eta isolamendu soziala dira (Liddle, 1987). Azkenik, sintoma kognitiboek barne hartzen dituzte ikaskuntza, memoria, arreta eta kontzentrazio zailtasunak, eta baita disfuntzio exekutiboak ere (Joyce eta Roiser, 2007).

### 1.1.3 Eskizofreniaren etiologia

Eskizofrenia faktore etiologiko ugarik eragindako gaixotasun konplexua da. Faktore genetikoak eta ingurumen baldintzak eskizofreniaren hasieran eta garapenean eragiten dute (Sullivan et al., 2012; McCutcheon et al., 2020). Gainera, neurogarapenean oinarritutako hipotesi etiologikoa ere proposatu da, garuneko nahasmendu estruktural, funtzional eta neurokimikoekin loturik dagoena. Aldaketa horiek hainbat neurotransmisio-sistema eta zirkuituei eragiten diete, eta badirudi eskizofrenia pairatzen duten pazienteetan horien desoreka ematen dela. Ebidentzien arabera, eskizofreniaren patologian dopamina (DA), serotonina (5-HT) eta glutamato neurotransmisio-sistemetan desorekak agertzen dira. Hala ere, badirudi hipotesiok ez direla dela nahikoak eskizofrenia gaixotasuna bere osotasunean azaltzeko.

### 1.1.3.1 Asaldura genetikoak

Hainbat ikerketa epidemiologikok frogatu dute osagai genetikoa eskizofrenia gaixotasunaren garapenarekin erlazionaturik dagoela, %80 inguruko heredagarritasunarekin (Sullivan et al., 2003). Azken urteotan, tamaina handiko azterketa genomikoek ahalbidetu dute gaixotasunean inplikaturik dauden geneen deskribapena. Horietan oinarrituz, eskizofrenia gaixotasun poligeniko gisa deskribatu da.

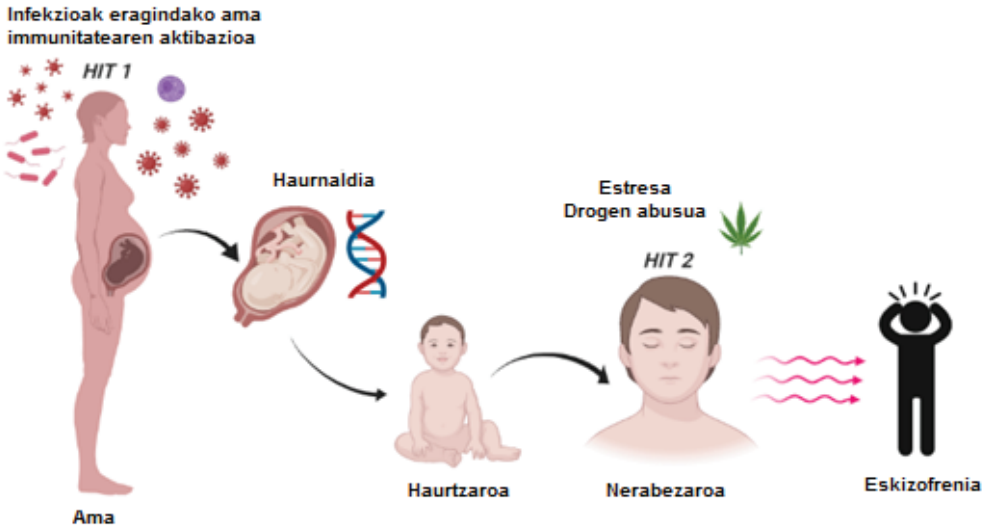
Genoma-osoaren asoziazio-azterketek (GWAS) frogatu dute aldagai genetiko desberdinak eskizofreniarekin erlazionaturik daudela (*Schizophrenia Working Group of the Psychiatric Genomic Consortium,* 2014). Errepikatzen diren gene asoziazioen artean aipatzekoak dira dentsitate postsinaptikoko proteinak (PSD), jarduerak erregulatzen dituen zitoeskeletoari lotutako proteinak, N-Metil-D-aspartato (NMDA) hartzailea, X adimeneko atzerapen proteina hauskorrak, eta neurogarapeneko beste alterazioak; besteak beste; kaltzioa ($Ca^{2+}$), zelula neuronalen atxikipen molekulak eta 2 motako dopamina hartzailea ($D_2R$) (Pardiñas et al., 2018, Trubetskoy et al., 2022). Gainera, histobateragarritasun konplexuarekin erlazionaturiko geneak ere eskizofrenia gaixotasunarekin lotu izan dira (Sekar et al., 2016).

### 1.1.3.2 Ingurumen faktoreak

Ingurumen faktoreak eskizofreniaren etiologian eragin zuzena dute. Hainbat ikerketa proposatu dute faktore genetikoez gain ingurumen faktore desberdinek gaixotasunaren agerpena bultzatu dezaketela, aldez aurretiko joera genetikoa duten pertsonetan (Van Os et al., 2010).

Ingurumen-faktoreak eskizofreniaren neurogarapenaren hipotesiarekin erlazionatu dira batez ere (Fatemi eta Folsom, 2009). Hainbat azterlanen arabera, haurdunaldiaren neurogarapen goiztiarrean ematen diren eragozpenak, eskizofreniaren intzidentzia arriskua areagotzearekin

erlazionatuak daude. Neurogarapenean eragin dezaketen faktoreen artean amaren estresa, amari eragiten dioten infekzioak, nutrizio-defizitak eta erditze eragozpenak aurkitzen dira, besteak beste (**1.1 irudia**) (Jones et al., 1998; McGrath et al., 2010; Marrón, 2012). Gainera, haurtzaroan pairatutako ezbeharrek eskizofrenia garatzeko arriskua handitzen dutela ere frogatu da (Varese et al., 2012). Bestalde, ondo ezarritako ebidentziek erakusten dute faktore sozioekonomikoek (Allardyce eta Boydell, 2006) eta immigrazioak (bai lehen belaunaldikoak, baita bigarren belaunaldikoek ere) eskizofrenia garapenarekin zerikusia dutela (Cantor-Graae eta Selten, 2005). Substantzia toxikoen kontsumoak ere badu eragina eskizofreniaren garapenean. Izan ere, hainbat ikerketen arabera bada lotura bat kalamua kontsumoaren eta psikosiaren artean (**1.1 irudia**). Azterlan horien arabera, kalamuaren esposizio kroniko goiztiarrek gertaera psikotikoak edukitzeko aukerak handitzen dituzte, baita eskizofreniaren ondorengo garapenean eraginak ere (Moore et al., 2007; Ibarra-Lecue et al., 2018) (**1.1 irudia**). Hori horrela, badirudi gene konkretuak eta inguruneko arrisku-faktore asko oso estuki lotuta daudela eskizofreniaren garapenarekin.




**1.1 Irudia**: Hainbat ingurumen faktorek izan dezaketen eragina eskizofreniaren garapenean; irudikapen eskematikoa. Haurdunaldiko infekzioek zitokina-proinflamatorioak askatzea eta immunitate-sistema aktibatzea eragiten dute. Aurrekari genetikoek, egoera autoimmuneak eta haurtzaroko eta nerabezaroko egoera desberdinek (estresa eta droga-abusua barne), amaren infekzioaren ondorioekin konbinatuta, handitu egiten dute etorkizunean eskizofrenia garatzeko probabilitatea. N Corderok tesi honetarako sortutako ilustrazioa eta *Estes eta McAllister-etik* egokitua (2016).

## 1.1.3.3 Eskizofreniaren neurotransmisio-sistemen alterazioak

### Eskizofreniaren hipotesi dopaminergikoa

Sintoma klinikoak eta eskizofreniaren farmakoen erantzuna azaltzeko teoriarik ezagunena hipotesi dopaminergikoa da, eta hainbat aurkikuntzatan oinarritzen da. Alde batetik, antipsikotiko tipikoen eragin klinikoak bide mesolinbiko kortikaleko $D_2R$ dopaminergikoen blokeoan oinarritzen dira. (Seeman eta Lee, 1975). Bestetik, dopamina mailak handitzen dituzten drogek, hala nola anfetaminak, gertakari psikotikoak eragiten dituzte gizabanako osasuntsuetan, eta psikosia areagotzen dute paziente eskizofrenikoetan (Lieberman et al., 1987).

*Sarrera*

Hipotesi dopaminergikoaren arabera, sintoma positiboak (haluzinazioak, eldarnioak) garuneko eremu mesolimbiko-estriatalean transmisio dopaminergikoaren hiperaktibitatearen ondorio izango lirateke. Hala ere, sintoma negatiboak eta kognitiboak antipsikotikoekiko erresistenteak dira. Hori dela eta, hipotesi dopaminergikoa birformulatu egin zen, hipodopaminergia kortikala proposatuz, eta hori sintoma negatibo eta kognitiboekin erlazionatuz (Davis et al., 1991; Howes eta Kapur 2009; McCutcheon et al., 2020).

Dopamina hartzaileak G-proteinei lotutako hartzaileak (GPCR) dira. Bi familia nagusitan sailkatu daitezke: $D_1$ dopamina-hartzaileen familia, $D_1$ eta $D_5$ dopamina-hartzaileak barne hartzen dituena ($D_1$R, $D_5$R), eta $D_2$ hartzaileen-familia, $D_2$, $D_3$ eta $D_4$ dopamina hartzaileak barne hartzen dituena ($D_2$R, $D_3$R, $D_3$R). $D_2$Ren blokeoa antipsikotikoen ekintza mekanismo nagusia da, gehienbat antipsikotiko tipikoena. Hori horrela, hartzaile dopaminergikoak funtsezko zeregina dutela proposatu da eskizofrenian.

*In vivo* eginiko neuroirudi azterketak, positroien igorpen bidezko tomografia (PET) eta fotoi bakarraren igorpen bidezko tomografia konputarizatua (SPECT) erabiliaz, besteak beste, eskizofrenia duten pazienteen $D_2$R eta $D_1$R hartzaileen egoera ebaluatzeko erabili dira. Hasierako azterketek, emaitza kontrajarriak aurkitu zituzten; batzuek $D_2$Ren igoera adieraziz, eta beste batzuek, berriz, kontrolekin alderik ez zutela (Howes et al., 2012, Cumming et al., 2021). $D_2$Ren dentsitate altua, erregulazio mekanismoak direla eta, tratamendu antipsikotiko kronikoaren ondorio zela iradoki zen. Aldiz, farmakorik jaso ez zuten paziente eskizofrenikoek ez zituzten $D_2$Ren igoerak aurkeztu, hartzaileen dentsitatearen igoera eta tratamendua erlazionatzen zituena (Seeman, 2013). Aurreko emaitzak indartu egin ziren *in vitro* post-mortem giza garunean $D_2$Ren handipena azaldu ondoren (Seeman et al., 1984, Zakzanis eta Hansen, 1998). Gainera, duela gutxi argitaratutako azterlan baten arabera, post-mortem garun eskizofrenikoetan ez dago $D_2$Ren aktibitatearen handipena (Eguskiza et al., 2021). $D_2$R-rekin bezala, *in vivo*

azterketek eskizofrenikoen aurre-garunazalean $D_1$Rren dentsitateari buruzko emaitza kontrajarriak aurkitu zituzten, aurretik tratamendurik gabeko pazienteetan (Cumming et al., 2021). Azkenengo ikerketak, berriz, $D_1$R eta $D_2$R postsinaptikoen egoeran baino neurotransmisio dopaminergiko parasinpatikoa aztertzera zuzendu dira. Hala, DA sintesi presinaptikoa (Howes et al., 2012; Fusar-Poli eta Meyer-Lindenberg, 2013), DA kontzentrazio sinaptikoa (Abi-Dargham et al., 2000; Caravaggio et al., 2015) eta anfetaminak eragindako DA askapena (Howes et al. 2012; Laurelle, 1998) handiagoa frogatu da paziente eskizofrenikoen estriatumean. Bestalde, eskizofrenia duten pazienteen aurre-garunazalean anfetaminak eragindako DAren askapenaren murrizketa ere frogatu da, eta horrek hipodomapinergia kortikal presinaptikoa dagoela iradokitzen du aurre-garunazalean (Slifstein et al., 2015).

## Eskizofreniaren hipotesi glutamatergikoa

Glutamatoa garuneko neurotransmisore kitzikatzaile nagusia da, eta hartzaile ionotropiko eta metabotropiko hartzaileekin elkarrreragiten du. Batetik, hartzaile ionotropikoen taldeak NMDA, kainatoa eta α-amino-3-hidroxi-5-metil-isoxazol-4-propionato (AMPA) hartzaile azpimotak biltzen ditu. Bestetik, glutamatoaren hartzaile metabotropikoak (mGluR) G-proteinaren bidezko seinalearen transdukzioa aktibatzen dute, eta I (mGlu1, mGlu5), II (mGlu2, mGlu3) eta III (mGlu6, mGlu4, mGlu7, mGlu8) taldeetan banatzen dira (Naka, 1992; Niswender eta Conlun, 2010; Muguruza et al., 2016).

Ondo ezarrita dago NMDA hartzaileen antagonisten administrazioek, hala nola fentziklidina (PCP) edo ketamina, psikosiaren antzeko egoerak eta defizit kognitiboak eragin ditzaketela gizaki osasuntsuengan. Ketamina eta PCPa NMDA hartzaileen antagonista ez-lehiakorrak dira (Thomson et al., 1985). Aurkikuntza horietan oinarrituta, NMDA hartzaileetan izaten den desorekak

eskizofreniarekin erlazioa izan dezakeela uste da (Javitt eta Zukin, 1991; Stone et al., 2008). Bitxia bada ere, anfetamina edo beste agonista dopaminergiko bat, NMDA hartzaileen antagonistekin batera administratzean sintoma kognitibo eta negatiboak sortzen ditu, eskizofreniaren fisiopatologia imitatuz (Krystal et al., 2005). Hori dela eta, NMDA hartzaileen antagonista ez lehiakorren administrazioa animalia eredu gisa erabiltzen da eskizofrenia aztertzea helburu duten ikerlanetan.

Horrekin lotuta, hainbat ikerketatan paziente eskizofrenikoen glutamato mailak neurtu dituzte, sintoma mota desberdinekin erlazionatzeko helburuarekin. Horrela, sistema glutamatergikoaren desorekak eskizofreniaren disfuntzio kognitiboekin erlazionatu dira (Moghaddam eta Javitt, 2012). Izan ere, aurretik azaldutako hiperdopaminergia estriatala eta hipodopaminergia kortikala hipotesi glutamatergikoarekin erlazionatu dira. Lehenengo, NMDA hartzaileen hipoaktibitatea edo inhibizioa deskribatu da eremu dopaminergiko mesolinbikoetan, azido-aminobutirikoa (GABA) askatzen duten interneuronetan. Horrek, egoera toniko inhibitzaileari kalte egingo lioke, eta, ondorioz, dopaminaren sintesi eta askapen handiagoa ekarriko luke, sintoma psikotikoak eraginez. Eta alderantziz, sintoma negatiboak NDMA hartzaileen hipofuntzioak eragindako hipoaktibitate dopaminergiko kortikalarekin erlazionatu dira (Javiit, 2010).

## Eskizofreniaren hipotesi serotonergikoa

Eskizofreniaren hipotesi serotonergikoa azido lisergikoaren D-dietilamida (LSD) droga haluzinogenoaren eta 5-HTren (Freedman 1961) arteko interakzioei buruzko lehen ikerketetatik sortu zen. LSDak eta bestelako droga haluzinogenoek eskizofreniaren sintoma positiboen antzerakoak diren buru nahasmenak eragiten dituzte, serotonina hartzaileak aktibatuz. Gainera, droga psikodelikoen egitura kimikoa 5-HT neurotransmisorearen antzerakoa da. 5-

HT hartzaile azpimota guztien artean, ebidentzia ugarik adierazten dute haluzinogenoek serotonina 2A hartzaile azpimotarekin (5-$HT_{2A}$R) lotzen direla, beren eraginak sortzeko (González-Maeso et al., 2007; Geyer eta Vollenweider 2008; González-Maeso eta Sealfon, 2009; Madsen et al., 2019). Bestalde, farmako antipsikotiko atipikoek 5-$HT_{2A}$Rekiko afinitate handia erakusten dute, eskizofrenian gehien aztertu den itu farmakologikoa bilakatuz (Meltzer et al., 1989). **1.4 atalean** 5-$HT_{2A}$Ren fisiologia eta eskizofrenian duten inplikazioaren berrikuspen zehatzagoa aurki daiteke.

### 1.1.4 Garuneko alterazio morfologikoak

Aurre-garunazala eskizofreniaren sintoma positibo eta kognitiboekin erlazionatu da (Kolk eta Rakic, 2022). Izan ere, garuneko eskualde horretan eman daitezkeen lesioak, animalietan eta gizakietan, epe luzerako ondorioak eragin ditzakete; hala nola lan-memoriaren alterazioa, hautapen inpultso murriztua, ingurumen-estimulu handiagoak sumatzea eta jokabide-murrizketak. Beraz, neurri handi batean aurre-garunazaleko anomaliak eskizofreniarekin eta beste gaixotasun psikiatriko batzuekin lotu dira, sintoma amankomunak dituzten heinean (Xu et al., 2019).

Ondo deskribatuta dago eskizofrenia pairatzen duten pazienteen alboko bentrikuluaren %25eko zabaltzea ematen dela, garunaren bolumen osoaren %2ko murrizketarekin (Johnstone et al., 1976; Haijma et al., 2013). Bentrikuluen tamaina gutxika handitzen da gaixotasunaren hasieratik, garunaren materia grisa murriztu egiten den heinean (Erp et al., 2016). Eskizofrenia duten pazienteen garunen post-mortem azterketa morfologikoek desberdintasunak erakutsi dituzte banaketa zelularrean; ziurrenik garunaren garapen goiztiarrean emandako migrazio neuronalaren alterazioekin, zelula piramidalen galerarekin, egitura zelular deformatuarekin eta GABA interneuronen kopuruaren murrizketarekin lotuak (Schmidt eta Mirnics, 2015).

## 1.2 G-proteinei loturiko hartzaileak (GPCR)

### 1.2.1 Ezaugarri orokorrak

GPCR edo 7 domeinuko mintz zeharreko hartzailea (7TM), gorputzeko mintz hartzaile familia azpitalde nagusia osatzen dute. Hartzaileak ligando mota desberdinak, hala nola ioiak, molekula txikiak eta peptidoak finkatzeko edo lotzeko ahalmena dute, zelulaz kanpoko seinaleak zelularen barnera transmititzeko (Alexander et al., 2019).

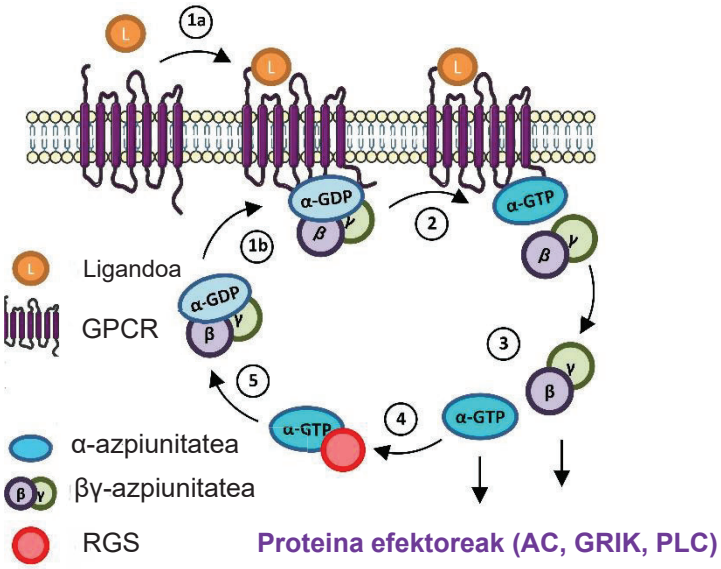
GPCRek garrantzi biomediko handia dute, hainbat jarduera fisiologiko zein patologiatan parte hartzen baitute. Gainera, farmakoen ekintza mekanismoa eragiteko itu nagusiak dira, merkaturatutako farmakoen %35a horrelakoa izanik (Hauser et al., 2017).

7TM hartzaileen superfamiliari GPCR ere deitzen zaie, beren ekintza G-proteinen bitartez betetzen dutelako. G-proteinak guanina nukleotidoak lotzen dituzten hiru azpi unitatez osaturik daude: α, β eta γ. Hala ere, GPCRak G-proteinekin lotzeaz gain beste proteina zitoplasmatikoetara lotu daitezke, β-arrestinetara besteak beste (Syrovatkina et al., 2016). G-proteinen aktibazioa, hortaz, zelulaz kanpoko seinalea zelularen barneko erantzun fisiologikoan bilakatzeko lehen urratsa izango da (Sriram eta Insel, 2018). Modu horretan, G-proteinak transduktore edo GPCRek sorturiko anplifikadore gisa jokatzen dute, zelula barruko erantzuna sortzeko.

### 1.2.2 GPCRen seinaleztapenaren oinarrizko mekanismoa

G-proteina heterotrimerikoek funtsezko zeregina dute erantzun zelularren espezifikotasuna eta ezaugarriak definitzeko. Ligandoen bidezko aktibazioaren ondorioz, GPCRen konformazio aldaketa gertatzen da, G-proteinekiko afinitatea areagotzen duena, eta G-proteinen erreklutamendua eragiten duena. Ligando, hartzaile eta G-proteinen arteko elkarrekintzak G-

proteina heterotrimerikoaren α-azpiunitateari loturik dagoen guanosina difosfato (GDP) nukleotidoa askatzea dakar, eta guanosina trifosfato (GTP) nukleotidoarekin trukatzea sustatzen du. GDP-GTP trukeak proteina heterotrimerikoa osatzen duen azpiunitateen eta hartzaileen arteko disoziazioa eragiten du. Egoera horretan, Gα eta Gβγ azpiunitateak hainbat efektoreekin elkarrekintza izaten dute, ligandoaren erantzuna zelula barnera transduzitzeko; hala nola, adenilato ziklasa (AC) fosfolipasa eta erreten ionikoak (Gilman, 1987; Hamm, 1998; Marinissen eta Gutkind, 2001). Azpiunitate horien seinaleztapena amaitzeko, eta prozesu haori etengabe gertatzeko, G-proteinak bere forma heterotrimerikora bueltatu beharra du. Horretarako, Gα-azpiunitatearen GTPasa aktibitateak GTP nukeotidoa GDPra hidrolizatzen du, Gα-azpiunitatea Gβγ dimeroaren elkarketa ahalbidetuz, eta G-proteina inaktiboa osatuz berriro (Milligan eta Kostenis, 2006; Hilger et al., 2018) (**1.2 Irudia**).




**Irudia 1.2**: GPCR/G-proteina aktibazio ereduaren irudikapen eskematikoa. Atseden-egoeran edo egoera inaktiboan, G-proteina heterotrimeroak dira, eta osaturik daude Gα azpiunitateaz (GDPrekin elkartuta) eta Gβγ azpiunitateaz (**1a**). Ligando baten aktibazioaren ondoren (neurotransmisore gisa), GPCRak aldaketa konformazionala sufritzen du, G-proteinetara lotzea ahalbidetzen duena (**1b**) eta GDP GTPaz trukatzea sustatzen du Gα azpiunitatea Gβγ azpiunitatearekin banatuz (**2**). Momentu horretan, G-proteina egoera aktiboan dago α eta βγ azpiunitateek zelula barneko efektoreekin elkarreragingo dute, hainbat seinaleztapen bide modulatzeko ahalmenarekin (**3**). Seinaleztapena amaitzen da GTP molekula GDPra hidrolizatzean, G-proteina erregulatzaileen bitartez (RGS) (**4**). Azkenik, konplexu heterotrimerikoa osatzen da, G-proteinak egoera inaktibora bueltatuz (**5**). *Oliveria et al., 2019ren* ilustrazio egokitua.

Agonistak GPCRarekin elkartzeak, G-proteinak estimulatzen dituzten konformazio-aldaketak abiarazteaz gain, G-proteinei lotutako kinasen (GRK) bidezko hartzailearen fosforilazioa errazten dute. Fosforilazioak arrestinen erreklutamendua sustatzen du, GPCRen erregulazioa murrizteko eta G-proteinen mendeko seinaleztapena arintzeko. Horrez gain, β-arrestinen eta G-

proteinekiko independienteak diren seinaleztapenak aktibatu ahal dira, aktibatutako-mitogenoen-protein-kinasen (MAPK) bidea aktibatuz (Wang et al., 2018).

G-proteinekin eta β-arrestinekin bat egiteaz gain, GPCRak beste GPCRekin elkarreragin dezakete dimeroak eratzeko; baita goi-mailako oligomeroak ere, sarritan funtsezkoak direnak GPCRen funtzioa modulatzeko (Milligan et al., 2019).

## G-proteinak

G-proteina heterotrimerikoak orokorrean lau talde nagusitan sailkatzen dira, Gα azpiunitatearen arabera: $G\alpha_{s/olf}$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ eta $G\alpha_{12/13}$ (Simon et al., 1991). Hartzailearen aktibazioaren ondoren, Gα-proteina familia bakoitzak seinaleztapen-bide desberdinak aktibatzen ditu, hainbat erantzun fisiologiko eraginez (**1.3 irudia**).

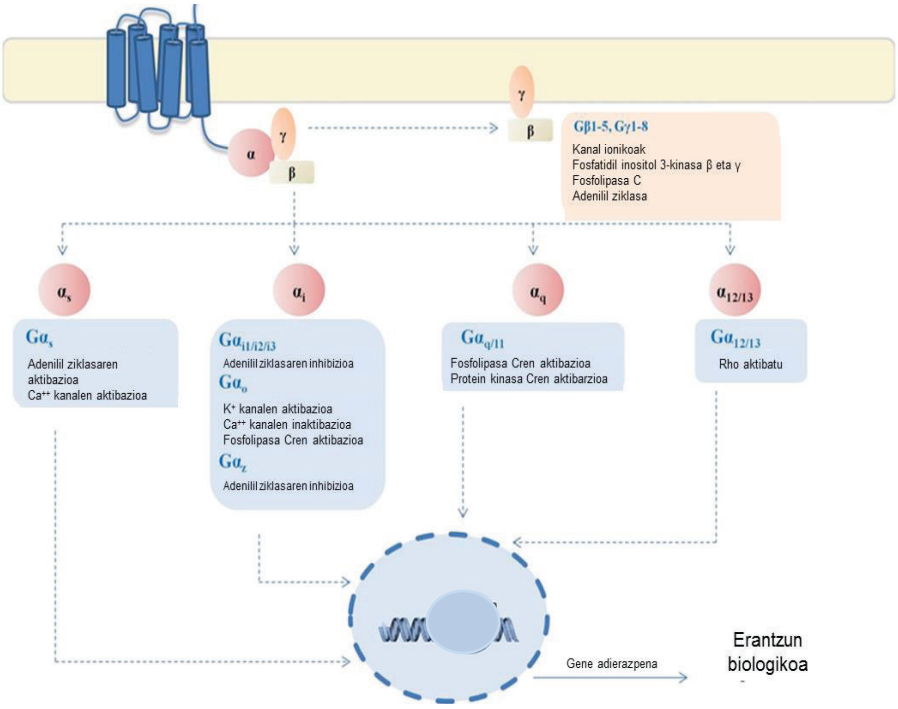
$G\alpha_s$ familia bi azpiunitatez osatuta dago: $G\alpha_s$-proteina, zelula gehienetan espresatzen dena, eta $G\alpha_{olf}$-proteina usaimen-neurona sentsorialetan espresatzen dena, batez ere (Weinstein et al., 2007). $G\alpha_s$ familiako proteinek, adenilato ziklasa (AC) estimulatzen dute, bere jarduera katalitikoa estimulatuz, eta, ondorioz, adenosina monofosfato ziklikoaren (cAMP) ekoizpena sustatzen du (Milligan eta Kostenis, 2006). Horrez gain, $G\alpha_{i/o}$ familiak AC inhibitzen du cAMP maila zelularrak murriztuz (Busnelli et al., 2013). $G\alpha_{i/o}$ familia $G\alpha_{i1}$-, $G\alpha_{i2}$-, $G\alpha_{i3}$-, $G\alpha_o$- eta $G\alpha_z$-proteina azpimota desberdinetan banatzen da. $G\alpha_i$ azpiunitateak zelula gehienetan espresatzen dira, garuna barne, eta %85-95 homologia partekatzen dute (Plummer et al., 2012). $G\alpha_o$ neuronetan espresatzen da, batez ere, eta garuneko G-proteina ugariena da (Sternweis eta Robishaw, 1984). $G\alpha_z$-proteinaren espresioa ehun neuronalean eta plaketetan mugatzen da (Hultman et al., 2014). Bukatzeko, $G\alpha_{i/o}$ familiako kide

guztiek ($G\alpha_z$ izan ezik) karboxilo terminalean ondo kontserbatutako zisteina hondakin bat dute. Eremu hori adenosina difosfatoaren (ADP) erribosilazio-guneari dagokio, G-proteinen aktibazioaren inhibizioa eragiten duena, *Bordetella pertussis toxina* (PTX) katalizatu ostean, (Morris eta Malbon, 1999). Ondorioz, proteina familia horiek PTX-sentikor gisa sailkatu ohi dira.

$G\alpha_{q/11}$ familiako kideek fosfolipasa β (PLC-β) aktibatzeko ahalmena dute, inositol 1,4,5-trifosfatoaren ($IP_3$) eta diazilglizerolaren (DAG) formakuntza bultzatuz, mintz plasmatikoan fosfatidilinositol 4,5-bifosfatotik ($PIP_2$) abiatuta. $IP_3$ak erretikulu endoplasmatikoan kaltzioa mugiarazten du, DAG proteina kinasa C (PKC) aktibatzen duen bitartean (Wettschureck et al., 2005; Wilkie et al., 2021). $G\alpha_{q/11}$ familia $G\alpha_q$-, $G\alpha_{11}$-, $G\alpha_{14}$- eta $G\alpha_{15/16}$ azpi-proteinez osaturik dago. $G\alpha_{11}$- eta $G\alpha_q$-proteinak edonon adierazten dira, eta aminoazidoen %88an sekuentzia partekatzen dute (Wilkie et al., 1991). Aldiz, $G\alpha_{14}$- eta $G\alpha_{15/16}$-proteinen espresioa ehun espezifikoetara mugatua dago; hala nola giltzurrunetara (Tanaka et al., 2000).

Azkenik, $G\alpha_{12/13}$-proteina familiak Rho guanina nukleotidoen aldaketa faktorea (RhoGEFs) estimulatzen du, eta bi azpi-familiatan sailkatzen da; $G\alpha_{12}$- eta $G\alpha_{13}$-proteinak, zelula mota gehienetan espresatzen direnak (Siehler, 2009).

Nahiz eta hasieran GPCR bakoitza mota bakarreko G-proteinek estimulatzeko ahalmena zuela uste uste izan, gaur egun frogatu da GPCRek G-proteina desberdinetara lotzeko ahalmena dutela, seinaleztapen bide desberdinak aktibatuz.




**1.3 irudia**: G-proteina desberdinen seinaleztapenaren irudikapen eskematikoa. Ilustrazioa: *Diez-Alarcia et al., 2016.*

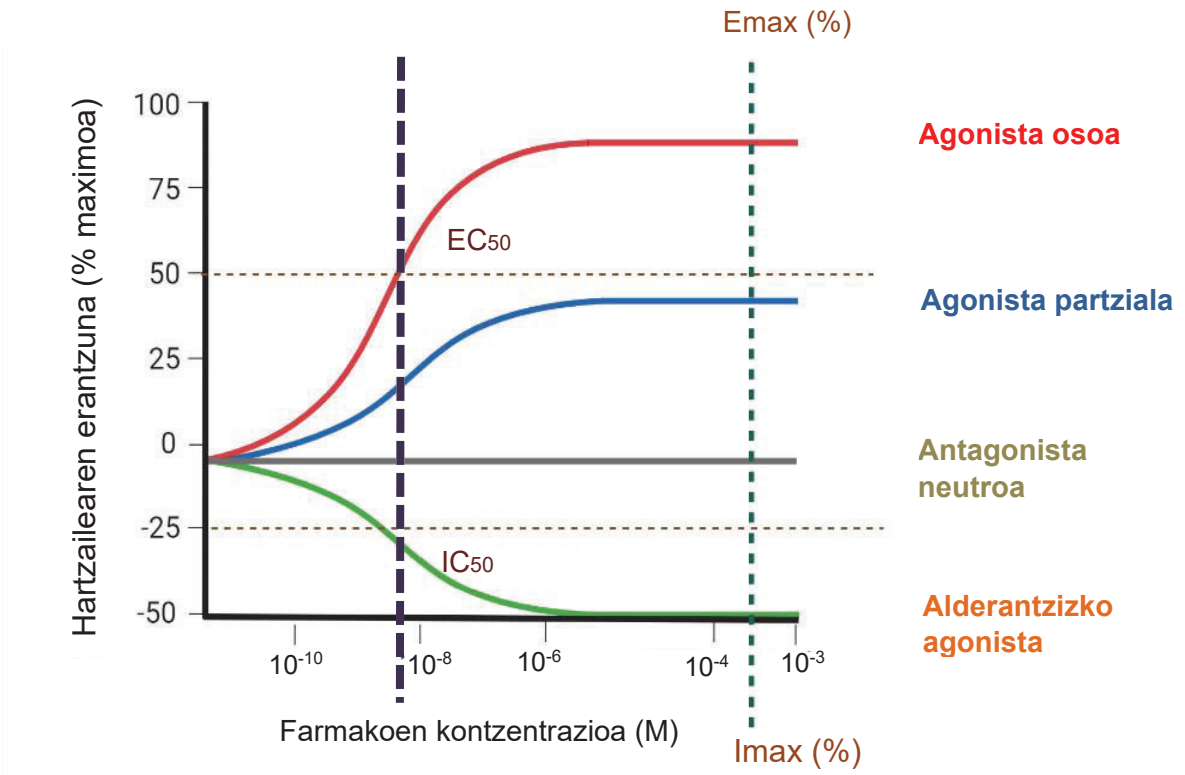
## 1.2.3 Farmako eta GPCR hartzaileen arteko elkarreraginen teoria

Farmakoek GPCRengan duten eragina funtsezko bi gertaeren araberako da: Lehenik eta behin, ligandoa hartzailearekin lotu behar da, eta hori farmako bakoitzaren afinitatearen araberakoa da. Bigarrenik, GPCR eta farmakoaren arteko loturak hartzailearen konformazio aldaketa eragin dezake, seinaleztapen-sistema batekin erlazionaturik dagoena, eta horri farmakoaren eraginkortasuna deritzo (Kenakin, 2002).

Afinitate farmakologikoa farmako bakoitza hartzaile espezifiko batekin elkartzeko edo finkatzeko duen gaitasuna da. Gainera, afinitatea oreka-disoziazioaren konstantearen alderantzizkoari dagokio ($1/K_D$); hau da, hartzaile kopuru osoaren %50a okupatzeko behar den farmakoaren

kontzentrazioari dagokio (Strange, 2008). Farmakoaren potentzia, aldiz, balio funtzional bati dagokio. Farmako bakoitzaren eragin terapeutikoa, ligando edo farmakoak hartzailearekin duen afinitatearen araberakoa da. Hori horrela, farmako bakoitzaren hautakortasunak bere eragin terapeutikoa mugatuko du. Horren arabera, medikamentuek eragin desiragaitzak eragiten dituzte, eragin terapeutikoarekin batera. Izan ere, eragin desiragaitzak, gehienetan, hautakortasun ezaren ondorio izaten dira (Roth et al., 2004).

Eraginkortasuna, gainera, zelula-inguruaren araberakoa izaten da. G-proteina bezalako transduktoreek, adibidez, GPCRen afinitate zelularra mugatzen dute. Eraginkortasuna, edo erantzun bat eragiteko ahalmena, efikazia intrintseko gisa ere ezaguna da. Hortaz, eraginkortasuna farmako batek hartzailea estimulatzean sortzen duen erantzun zelular gisa definitzen da (Kenakin, 1999). Farmako batek kontzentrazio jakin batean sortzen duen erantzun zelularra sistemaren mendeko ezaugarrien (hartzailearen dentsitatea eta hartzaileak efektoreekin eragiteko duen ahalmena) zein sistemarekiko independenteak eta farmako menpekok diren ezaugarrien mendeko da (Berg eta Clarke, 2018). Potentzia eta eraginkortasun espezifikoa dosi-erantzun kontzentrazio kurbak erabiliz neurtu daiteke (**1.4 irudia**). Batezbesteko efektu maximoa lortzeko % 50 kontzentrazioa ($IC_{50}$/$EC_{50}$) zein estimulazio/inhibizio efektu maximoen ($I_{max}$/$E_{max}$) balioak lortu daitezke, potentzia eta eraginkortasunari dagozkien parametroak, hain zuzen ere (Strange, 2008; Wacker et al., 2017). Iraganean, farmako bakoitzak ligando endogenoarekin konparatzean erantzun bat sortzeko duen ahalmenaren arabera sailkatu ziren: agonista osoak, agonista partzialak eta antagonistak. Berriki, aldiz, alderantzizko agonismo kontzeptua sortu zen, antagonista neutral gisa jokatzen ez zuten antagonista teorikoen ezusteko erantzun funtzionalak azaltzeko.




**1.4 irudia**: GPCRen kontzentrazio-erantzun kurbaren irudikapen eskematikoa. Y ardatzean, ehunekotan hartzaileen erantzuna zehazten da, erantzunaren %100 erantzun maximoa izanik eta %0 erantzun eza. X ardatzak ligandoaren kontzentrazio desberdinak adierazten ditu. %0a ligando ezean hartzaileak berez duen aktibitateari dagokio; hau da, aktibitate basal edo aktibitate konstitutiboari. Beraz, **agonista osoek** erantzun maximoa eragiten dute, aldiz, **agonista partzialek** ez dute erantzun maximora heltzeko ahalmenik. **Antagonista neutroek**, berezko eraginkortasun eza daukate, beraz, ez dute hartzailearen berezko erantzuna aldatzen. Aitzitik, **alderantzizko agonistek** berezko jarduera edo jarduera basala murrizteko edo inhibitzeko ahalmena dute. Erantzun maximo estimulatzaile edo inhibitzailea ($I_{max}/E_{max}$) ligando batek eragin dezakeen erantzun maximoari dagokio. Efektu maximoaren %50eko erantzuna lortzeko beharrezkoa den kontzentrazioa $IC_{50}$ edo $EC_{50}$ri dagokio.

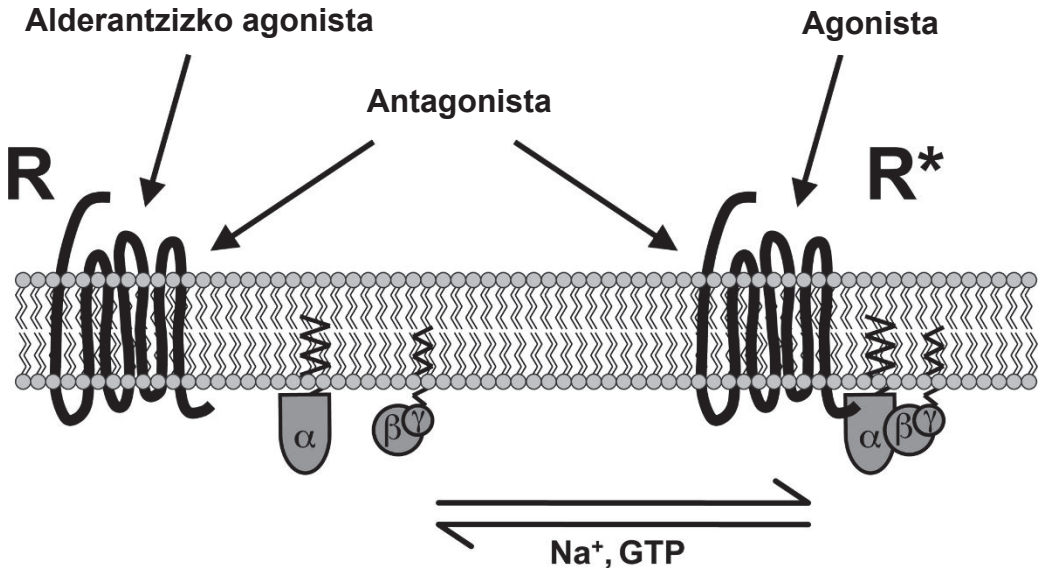
*Sarrera*

Farmakoek hartzaile jakin bat edo batzuekiko afinitatea eta eraginkortasuna izaten dute, beraz, hartzaileekin lotzeko eta, ondorioz, erantzun bat sortzeko ahalmena dute. Farmakoek hartzailearekin lotu ostean sortzen den erantzun maila desberdina izan daiteke, agonista oso edo partzial gisa sailkatuz. Agonista osoak erantzun maximoa sortzeko gaitasuna du, aldiz, agonista partzialak ez du erantzun osora heltzeko ahalmenik. Aitzitik, antagonistek hartzailearekiko afinitatea badute, baina ez dute berezko eraginkortasunik, ez baitute hartzailearengan konformazio aldaketarik eragiteko ahalmenik. Antagonistek duten ahalmena, ligando endogeno edo bestelako farmako batek sortzen duten erantzuna blokeatzeko edo murrizteko duten gaitasunaren araberako da.

Bestalde, Samama et al., lanean proposatzen da ligando edo farmako baten presentzia ez dela beharrezkoa erantzun zelularra edo seinaleztapen bideak aktibatzeko (Samama et al., 1993). Aktibitate konstitutiboa hartzaileak auto aktibatzeko eta seinaleztapena sortzeko ahalmenari dagokio, ligando ezean (Lefkowitz et al., 1993). Zentzu horretan, Costa eta Herzek identifikatu zituzten aktibitate konstitutiboa txikitzeko ahalmena zuten ligandoak, berezko erantzuna murriztueko gaitasuna zutenak (Costa eta Herz, 1989; Costa eta Cotecchia, 2005). Farmako edo ligando horiek alderantzizko agonista deitu ziren, eta sortzen duten erantzunaren tamainaren arabera alderantzizko agonista oso edo partzial gisa sailkatzen dira, berezko eraginkortasunaren arabera.

Horren harira, konplexu hirutarraren eredua, GPCRen seinaleztapen eredu onartuena da (De Lean et al., 1980). Eredu horren arabera, hartzailea oreka dinamikoan dago konformazio ez-aktibo (R) eta aktiboaren (R*) artean. Horrela, antagonista neutroek afinitate bera izaten dute konformazio aktibo eta inaktiboekiko. Agonistek, berriz, konformazio aktiboarekiko izaten dute afinitate handiagoa, agonistaren elkartzeak GPCRen oreka dinamikoa egoera aktibora bultzatzen duelarik, Rtik R*ra aldatuz.

Agonista baten eragin maximoa (eraginkortasuna), agonistak konformazio aktibo ($R^*$) eta konformazio inaktiboarekiko (R) duen afinitate desberdintasunarekin loturik dago. Aitzitik, alderantzizko agonistek afinitate handiagoa izaten dute hartzaile ez-aktiboarekiko (R), oreka dinamikoa R*tik R-ra aldatuz. Hala ere, alderantzizko agonisten eraginkortasuna, hartzailearen aktibitate konstitutiboaren araberakoa da (**1.5 irudia**).




**1.5 irudia**: Agonista, antagonista eta alderantzizko agonistak eta GPCRen egoera funtzional desberdinen arteko loturaren irudikapen eskematikoa. Agonistak hartzailearen egitura aktibora (R*) afinitate handiagorekin lotzen dira. Alderantzizko agonistek, berriz, egoera ez aktiboarekin elkartzeko eta egonkortzeko ahalmena daukate (R). Antagonista neutroek GPCRen egoera aktibo eta inaktiboarekiko afinitate bera dute. Ilustrazioa: *Muguruza et al., 2013.*

Hedatutako konplexu hirutarraren ereduaren arabera, hartzaileek egoera aktibotik egoera inaktibora aldatzeko ahalmena dute ligandorik gabe (Samama et al., 1993). Zentzu honetan, alderantzizko agonisten ezaugarri farmakologikoak agonisten ekintzen blokeoan zein aktibitate basalaren gutxipenean oinarritzen dira. Ondorioz, alderantzizko agonisten aktibitatea frogatzeko, oinarrizko hartzaileak aktibitate basala izan behar du,

alderantzizko agonistak aktibitate hori murriztu ahal izateko. Aktibitate konstitutiboa gutxitzea bi egoera desberdinetan desagertzen dela frogatu behar da alderantzizko agonista izaera egiaztatzeko; antagonistarekin batera jartzean eta parte hartzen duen hartzailearen *knock-out* animalietan. Alderantzizko agonisten ezaugarriak ezagutzean, iraganean antagonista neutro gisa ezagutzen ziren farmako asko alderantzizko agonista gisa jokatzen dutela frogatu da (Strange, 2002; Kenakin, 2004).

Hainbat ikerketek alderantzizko agonisten balizko erabilgarritasuna adierazi duten arren, farmako horien ezaugarri farmakologikoen erabilera terapeutikoari buruzko informazio gutxi dago. Badira hainbat gaixotasun hartzaileen aktibitate konstitutiboa handituta dutenak, hartzaileen mutazioen ondorioz; adibidez, gizonezkoen pubertaro goiztiar gaixotasunaren oinarrian, hartzaile somatikoen mutazioa duena (Kosugi eta Mori, 1995). Alderantzizko agonistak onuragarriak izan daitezke egoera horietan, mutazioek eragindako hartzailearen gehiegizko aktibitatea gutxituko luketelako. Kasu horietan, antagonista neutroak ez lirateke oso erabilgarriak izango, hartzailearen aktibitate handipena ez delako ligando endogenoaren ekintzaren ondorio (Ligt et al., 2000). Egoera kliniko horietan, aktibitate konstitutiboa detektatu edo deskribatu dezaketen metodo funtzionalak behar dira azterketak egiteko, eta alderantziko agonisten aurkikuntzan lagungarriak izan daitezke.

### 1.2.4 GPCRak eta seinalizazio alboratua

Aurretik aipatu bezala, GPCRak behin aktibatuak izatean, aldi berean seinaleztatu dezakete bide paraleloen bidez; hala nola G-proteina heterotrimerikoen, β-arrestinen edo GRK molekulen bitartez. Testuinguru horretan, deskribatu da ligando desberdinek, behin GPCR aktibatzean, seinaleztapen bide desberdinak aktibatu ditzaketela, hautakortasun funtzionala edo agonismo alboratua deituriko ezaugarriari esker (Perez eta

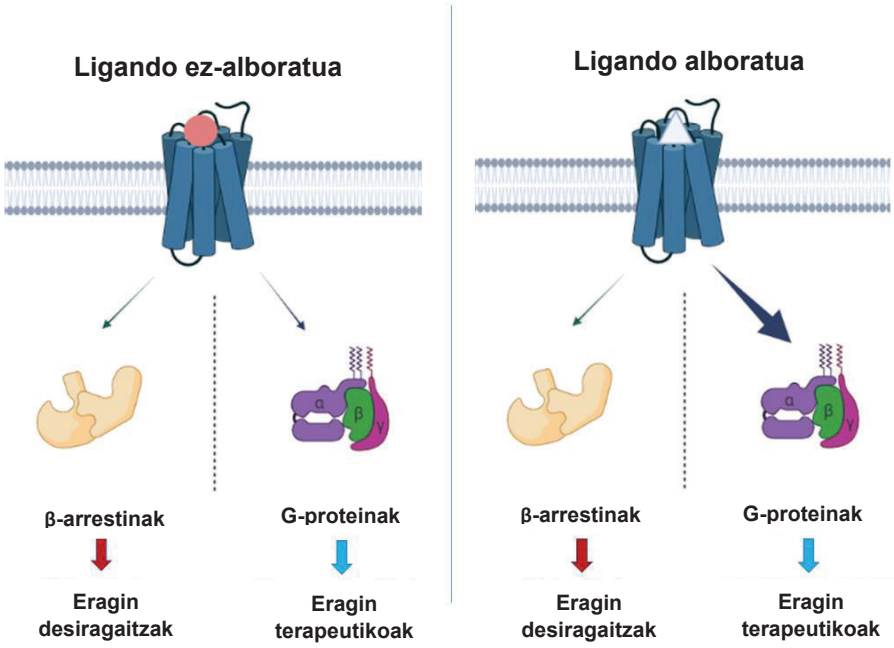
Karnik, 2005; Kenakin, 2011; Kenakin, 2012; Smith et al., 2018). Agonista alboratuak, teorian, GPCRen konformazio desberdinak egonkortzeko gai dira, eta, ondorioz, GPCRek seinaleztapen transduktoreekiko afinitate desberdina izango lukete. Horrela, farmako berak hainbat erantzun zelular sortzeko ahalmena izango luke. Aitzitik, ligando batzuk, seinaleztapen bide guztiak aktibatzeko ahalmena dute. Farmako hauek, ligando orekatuak edo ligando ez-alboratu moduan definitzen dira.

Bitxia bada ere, seinaleztapen bide jakin bat aktibatzea edo inhibitzea, hautakortasunaren ikuspuntutik, kontzeptu berria suposatzen du, eragin terapeutiko hobetuak eta eragin desiragaitz gutxiago sortzen dituzten farmakoak diseinatzea ahalbidetzen duena. Horrela, seinaleztapen bide jakin baterako hautakortasuna aurkezten duten ligandoak garatzeak, hainbat hartzaile espezifikorentzat, gaur egungo medikamentuen garapenean erronka nagusietakoa da (Komatsu et al., 2019).

Opiodeen farmakologia da hautakortasun funtzionalaren adibide ezagunena (Che et al., 2021). Hautakortasun funtzionalak aktibazio diferentziala dakar, $G\alpha_i$-proteina eta β-arrestinen artean. Ebidentziak adierazten du, hartzaile opioideen eragin terapeutikoa, analgesia barne, $G\alpha_i$-proteinen aktibazioaren bitartez ematen dela. Eragin desiragaitzak, aldiz, arnas depresioa eta idorreria adibidez, β-arrestinen erreklutamenduarekin erlazionatu dira (Bohn et al., 1999). Ildo horretan, efektu terapeutiko hobetuak dituzten ligando opioide berriak garatu dira. Duela gutxi, TRV130 (oliceridina), $G\alpha_i$-proteinekiko *in vitro* hautakortasun funtzionala frogatu da, eta minaren tratamendurako segurua dela ikusi da, III. faseko azterketa kliniko baten arabera (Singla et al., 2019). Hala ere, beste ikerlan batzuek adierazi dute, ligando opioide alboratuen garapena eztabaidagarria dela, eta ikerketa gehiago behar direla farmako bakoitzaren seinaleztapen bideak zehazteko (Gillis et al., 2020; Kliewer et al., 2020).

Farmakoak garatzerako orduan, kontuan hartzeko beste alderdi bat, egitura kimikoak hautakortasun funtzionalean izan dezakeen eragina da (Shonberg et al., 2014). Adibidez, LDSak eta lisuride farmakoak *in vitro* eta *in vivo* erantzun desberdinak dituzte (González-Maeso et al., 2003, González-Maeso et al., 2007), nahiz eta egitura eta 5-$HT_{2A}$R-rekiko afinitate altua partekatu. Bestalde, risperidona eta bere metabolito aktiboa, paliperidona, talde hidroxilo bakar batean bereizten dira, eta biak farmako antipsikotiko atipikoak dira. Farmako horien ezaugarri farmakologikoak desberdinak dira, eta farmakoen hautakortasun funtzionalaren ezaugarrietan du jatorria desberdintasunak (Clarke et al., 2013). Aurkikuntza guzti horiek, egitura-hautakortasun funtzionalaren arteko erlazioaren azterketa sakonaren garrantzia azalarazten dute (Berg eta Clarke, 2018).

Nahiz eta ikerketa askok ligandoen hautakortasun funtzionalari buruzko informazioa argitu duten, ikerlan gehienak seinaleztapen bide jakin batzuetara mugatuta daude; hala nola G-proteinak edo β-arrestinen bideak elkarrekin alderatuz. Hala ere, beste seinaleztapen bide desberdinak ere aktibatu daitezke, eta horiek ere kontuan hartu beharko lirateke eraginkortasun ezaugarriak deskribatzerako orduan (**1.6 irudia**).




**1.6 irudia**: GPCRen hautakortasun funtzionalaren irudikapen grafikoa. μ-hartzaile opiodeen adibide hipotetiko eta sinplifikatua. G-proteinen aktibazio edo seinaleztapenak eragin terapeutikoa du; β-arrestinen aktibazioak, aldiz, eragin desiragaitzak sortzen ditu. Agonismo alboraturik gabeko ligandoek bi seinaleztapen bideak aktibatuko dituzte, eragin terapeutiko nahiz eragin desiragaitzak sortuz. Aldiz, agonismo alboratua aurkezten duten agonistek seinaleztapen bide bat aktibatzeko ahalmena dute, modu hautakorrean, eragin terapeutikoak sustatuz eta eragin desiragaitzik sortu gabe.

### 1.2.5 Aktibitate konstitutiboaren, alderantzizko agonismoaren eta hautakortasun funtzionalaren ebaluazioa

GPCRen inplikazioak hainbat gaixotasunetan handitu egin du hartzailei finkatzeko ahalmena duten ligandoak ikertzeko metodoen kopurua. Garrantzi handia dutenez, hainbat saiakuntza egin dira farmako desberdinen profil funtzionala zehazteko.

Saiakuntza funtzional klasikoak bigarren mezularien mailen neurketan oinarritzen dira; hala nola $Ca^{2+}$, $IP_3$ren metatzea edo cAMP ekoizpenean,

besteak beste. Saiakuntza horiek farmako bat GPCRarekin finkatu ostean duen profil funtzionala zehazteko baliagarriak dira. Bigarren mezulariak neurtzean, aldatu gabeko hartzaileak azter daitezke jatorrizko ehunetan. Bigarren mezularien neurketan oinarritzen diren saiakerak ikerkuntza desberdinetan erabili dira, eta horrek abantaila suposatzen du, argitalpenen artean emaitzak alderatzea ahalbidetzen duelako.

Bigarren mezularien neurketan oinarritzen diren teknikak asko erabiltzen diren arren, anplifikazio maila altua duten teknikak dira, eta, ondorioz, agonista partzialek eta agonista osoek desberdintzea zaila da; erantzun maximo bera izan dezaketelako (Smith et al., 2018). Gainera, ez da argi geratzen bigarren mezularien erantzuna zein G-proteina azpimotaren aktibazioaren menpekoa den. Hortaz, beharrezkoa da farmakoen eta G-proteinen arteko elkarreraginaren kuantifikazio funtzional hurbilagoa burutzea.

GPCR aktibazioaren ebaluazio zuzena egin daiteke, G-proteinetan ematen den guanina nukleotidoen trukearen estimulazioa edo inhibizioa neurtuz, erradioaktiboa eta ez-hidrolizagarria den GTP molekula erabiliz. Teknika honi 35 sufrearekin markaturik dagen guanosina-5´-O-(γ-ttio)-trifosfatoaren ([$^{35}$S]GTPγS) finkapen teknika esaten zaio. GPCR eta farmakoaren artean ematen den loturaren ondorioz gertatzen den G-proteinen erantzun funtzional goiztiarra neurtzen da, eta ez dago seinale-anplifikazioren menpe (González-Maeso et al., 2000; Harrison eta Traynor, 2003). [$^{35}$S]GTPγS finkapen teknika konbentzionala $G\alpha_{i/o}$-proteina eta GPCRen artean ematen den akoplamendua neurtzera mugatzen dira; nukleotidoen aldaketa tasa altuena eta aktibitate konstitutibo altuena duen G-proteinen familia delako $G\alpha_{i/o}$ (Seifert eta Wenzel-Seifert, 2002). Hala ere, Gα-proteina espezifikoak neurtzeko saiakuntza desberdinak garatu dira; hala nola, immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika (SPA) (Diez-Alarcia et al., 2021b). Teknika horrek [$^{35}$S]GTPγS finkapen teknika klasikoa G-proteina bakoitzaren kontrako antigorputzekin konbinatzen ditu. Metodologia kultibo zelularretara zein

jatorrizko ehunetara aplikatu daiteke, hartzaileetan aldaketarik eragin gabe. Gainera, teknika horrek aktibitate konstitutiboa neurtzea ahalbidetzen du, jatorrizko ehunean, tresna farmakologikoak (alderantzizko agonistak, adibidez) erabiliz (Diez-Alarcia et al., 2021b). Hala ere, immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika (SPA), Gα-proteinetara mugaturik dago. Hori horrela, β-arrestinen erantzuna ezin da oraindik neurtu.

Aurreko saiakuntzez gain, fluoreszentzia eta bioluminezentzia erresonantzia bidezko energia-transferentzian oinarritzen diren saiakuntzak garatu dira (FRET eta BRET). Proteinen arteko elkarrekintza eta konformazio-aldaketa dinamikoak detektatzeko teknologiak dira, GPCR, G-proteinak, eta β-arrestinen konformazio aldaketak zuzenean neurtzea baimentzen dutenak (Zhou et al., 2021; Wright eta Bouvier, 2021). Saiakuntza hauek, zelula bizien gertaerak denbora errelean monitorizatzea ahalbidetzen dute, eta errendimendu handiko detekziora egokitzeko aukera ematen dute. Teknika horiek abantailak izan arren, detekziorako erabiltzen diren molekula fluoreszenteek lortzen diren emaitzetan izan dezaketen eragina frogatu beharko litzateke (Pottie eta Stove, 2022). BRET teknikaren erabilera mugaturik dago *in vitro* zelula hazkuntza sistemetara, bizirik dauden animalietan ez baita erabilgarria. Gainera, oraingoz, erresonantzia bidezko energia-transferentzian oinarritzen diren teknikak ez dira erabilgarriak post-mortem ehuna aztertzeko (Drinovec et al., 2012).

## 1.3 Serotonina 2A hartzaileak (5-$HT_{2A}$R)

### 1.3.1 Orokortasunak

Nerbio sistema zentralean (NSZ) 5-HTk hainbat prozesu fisiologikotan parte hartze du, oroimena, pertzepzioa, kognizioa, emozioak, gogo aldartea eta kontzientzia, besteak beste (Berger et al., 2009). Sistema serotonergikoaren disfuntzioa gaixotasun psikiatriko askotan inplikaturik dago (Hoyer, 2020). Hartzaile serotonergikoak egitura eta ezaugarri farmakologikoen arabera, 14 azpimota desberdinetan sailkatzen dira (Hannon eta Hoyer, 2008), eta zazpi familia nagusi osatzen dituzte (5-$HT_1$, 5-$HT_2$, 5-$HT_3$, 5-$HT_4$, 5-$HT_5$, 5-$HT_6$, 5-$HT_7$ hartzaileak) (**1.7 irudia**). Egiturari dagokionez, 5-$HT_3$ hartzailea erreten ionikoa den hartzaile bakarra da (Maricq et al., 1991). Gainerako hartzaile serotonerikoak, aldiz, G-proteinetara loturiko hartzaile metabotropikoak dira (Kroeze eta Roth, 1998).

5-$HT_2$ hartzaileak gehien ikertu diren hartzaile serotonergikoen artean daude. 5-$HT_2$ hartzaileen familia 3 hartzaile azpimota desberdinetan banatzen da, 5-$HT_{2A}$R, 5-$HT_{2B}$R eta 5-$HT_{2C}$R, non %40-50 sekuentzia-homologia duten (Hoyer et al., 2002). Gainera, 5-$HT_{2A}$Ren eta 5-$HT_{2C}$Ren mintz arteko eremuek %80eko sekuentzia-homologia dute, eta antzekoak diren profil farmakologikoak partekatzen dituzte (Boess eta Martin, 1994). Hori dela eta, hartzaile bakoitzarentzat farmako hautakorrak garatzea funtsezko erronka da. Gaur egun, 5-$HT_2$ hartzaileen azpimota desberdinak farmakologikoki sailkatzea nahiko zaila da, ligando hautakorrik ez dagoelako.

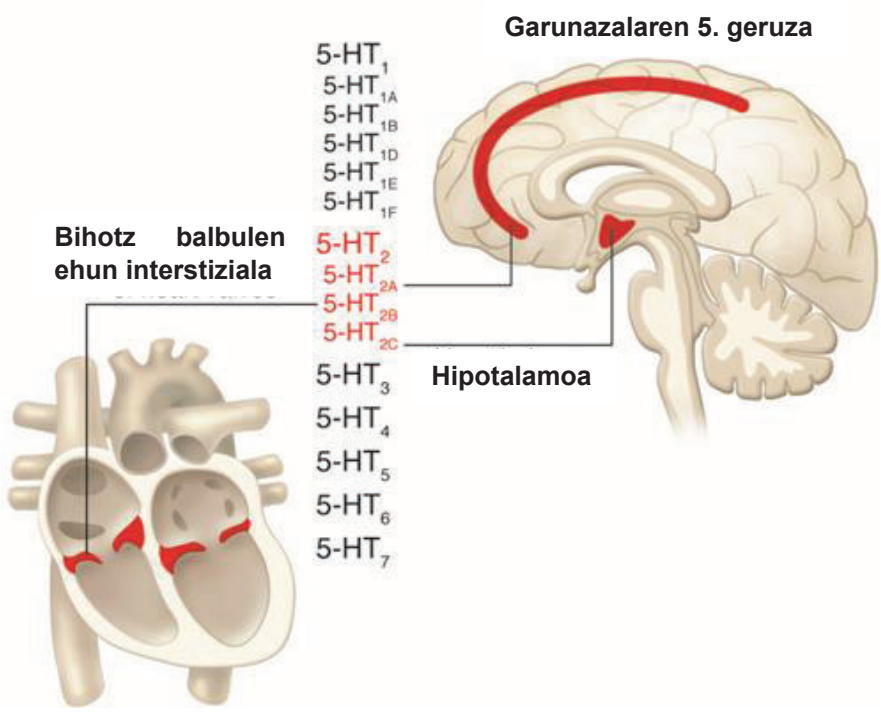
Hasieran batean, 5-HT azpimota desberdinak sailkatzeko erradioligandoen finkapen teknikak erabili ziren. Erradioligandoekin egindako lehenengo ikerketan oinarrituta, 5-HT molekularen bi finkapen eremu desberdin zeudela deskribatu ziren. [$^3$H]5-HT erradioligandoaren afinitate altuko gunea 5-$HT_1$ hartzailearen azpimotari dagokio. Afinitate baxuko guneak, berriz, 5-$HT_2$

hartzaile gisa sailkatu ziren. [$^{3}$H]ketanserina erradioligando hautakorraren aurkikuntza aurrerapen zientifiko izugarria izan zen, 5-HT$_{2A}$Rak ikertzeko (Leysen et al., 1982). Hala ere, ketanserinak 5-HT$_{2C}$Rekiko afinitatea aurkezten du, beste hartzaile batzuen artean (Choudhary et al., 1992). Azken urtetan, 5-HT$_{2A}$Rren azterketa egiteko ligando hautakor berriak garatu dira, [$^{18}$F]altanserina, [$^{3}$H]MDL100907 (L´Estrade et al., 2018) eta [$^{11}$C]Cimbi-36, besteak beste (Ettrup et al., 2014).

5-HT$_{2A}$R-ren eremu kodifikatzaileen sekuentzia analisiek espezien artean kontserbazio genetiko handia dagoela baieztatu zuten. Hala ere, gizakion 242 hondarrean serina bat aurkitzen bada ere, karraskarietan alanina bat aurkitzen da. Aldaketa horrek 5-HT$_{2A}$R agonisten afinitate eta eraginkortasunean eragina izan dezakeela proposatu da gainera (López-Giménez eta González-Maeso, 2018; Kim et al., 2020; Slocum et al., 2021).

### 1.3.2 5-HT$_{2A}$R kokapena eta funtzioa NSZean

5-HT$_{2A}$R eta 5-HT$_{2C}$R NSZean espresatzen dira biak, eta periferian, berriz, 5-HT$_{2A}$R plaketetan; muskulu-zeluletan eta begi ehunetan aurkitzen da (Leysen, 2004). Aitzitik, 5-HT$_{2B}$R batez ere periferian espresatzen da; zehazki, giza bihotz balbuletan (Bonaventure et al., 2005) (**1.7 irudia**).




**1.7 irudia**: Hartzaile serotonergiko azpimoten irudikapen eskematikoa (5-$HT_1$ - 5-$HT_7$). 5-$HT_2$ familia 3 hartzaile mota desberdinetan banatzen da (5-$HT_{2A}$R, 5-$HT_{2B}$R eta 5-$HT_{2C}$R). 5-$HT_2$ familiako hartzaile mota bakoitzak garunean eta bestelako ehunetan banaketa desberdina du. 5-$HT_{2A}$R gehien bat espresatzen da garun azaleko V geruzako neurona piramidaletan. 5-$HT_{2C}$R garunean ere espresatzen da, baina hipokanpoan. Azkenik 5-$HT_{2B}$R periferian, bihotzeko balbulen zeluletan dago. Ilustrazioa: *Meltzer eta Roth, 2013.*

5-$HT_{2A}$R-ren espresioa ikertzea posible izan da erradioligandoen finkapen teknika, immunohistokimika, mikroskopia elektronikoa eta *in situ* hibridazio teknikei esker. Bai post-mortem erradiografiak, bai *in vivo* neuroirudiaren ikerketek baieztatu ahal izan dute 5-$HT_{2A}$Raren espresioa giza garunean (Pazos et al., 1987; Forutan et al., 2002). Giza garunean gehientsuenat eskualde kortikaletan (batez ere, aurre-garunazalean, parietalean eta somatosentsorialean) espresatzen da. Eremu subkortikaletan eta hipokanpoan, berriz, dentsitatea baxuagoan aurkitzen da (Hoyer et al., 1986; Pazos et al., 1987; López-Giménez et al., 1997; Varnäs et al., 2004; Beliveau

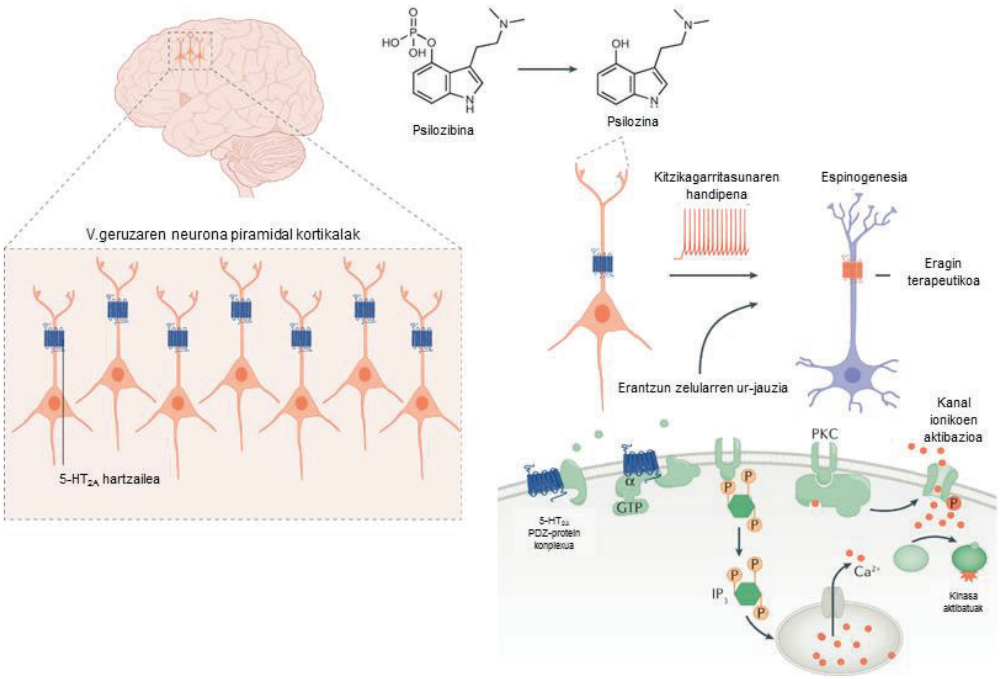
et al., 2017). Zehazkiago, post-mortem garunetan egindako ikerketa batek frogatu zuen 5-$HT_{2A}$R III eta IV geruza kortikaletan, hipotalamoan, eta neurri txikiagoan, hipokanpoan eta egitura estriataletan espresatzen dela (Pazos et al., 1987). Horrez gain, garunean aurkitzen den 5-$HT_{2A}$Ren espresioa adinarekin murriztu egiten da (Gross-Isseroff et al., 1990; González-Maeso et al., 2008; Moses-Kolko et al., 2011; Uchida et al., 2011; Muguruza et al., 2013; Diez-Alarcia et al., 2021a).

Gainera, 5-$HT_{2A}$R neurona glutamatergiko kortikaletan aurkitzen da, zehazki dendrita apikaletan (Jakab eta Goldman-Rackic, 1998; Miner et al., 2003; Santana et al., 2004). Interneurona GABAergikoetan ere espresatzen da (Burnet et al., 1995). Orokorrean, 5-$HT_{2A}$R sinapsi ondoko eremuetan kokatzen da, hartzaile post-sinaptiko moduan (sinapsi glutamatergikoei dagokionez) (Jakab eta Goldman-Rakic, 1998). Hala ere, 5-$HT_{2A}$R hein batean eremu presinaptikoan ere kokatzen dela uste da, neurona monoaminergikoen axoietan, hain zuzen (Miner et al., 2003; Bécamel et al., 2017). Gainera, 5-$HT_{2A}$R astrozitoetan eta mikroglian ere identifikatu da (Krabbe et al., 2012; Martin eta Nichols, 2016). Ugaztunen garunean, 5-$HT_{2A}$R espresioa handiagoa da frakzio zitosolikoetan mintz plasmatikoan baino (Cornea-Hebert et al., 1999; Eastwood et al., 2001). Mintz zitoplasmatikoan espresatzen den hartzailea G-proteinetara loturik dagoela uste da, mintzetik barneraturiko hartzailea ez bezala.

5-$HT_{2A}$R $G\alpha_{q/11}$-proteinetara lotzen da gehienbat, eta behin aktibatuta dagoela,PLC isoforma aktibatzen du, $IP_3$k ekoizteak $Ca^{2+}$ren askapena eragiten du, eta azkenik, PKC aktibatzen da (ikusi **1.3.4 atala** informazio gehiagorako) (**1.8 irudia**). *In vitro* esperimentuei esker frogatu da 5-$HT_{2A}$Ren aktibazioaren ondorioz aurre-garunazaleko neuronen kitzikagarritasuna handitzen dela (Araneda eta Andrade, 1991). Gainera, agonisten administrazio kortikalak kitzikagarritasun neuronala eta neurotransmisoreen askapena handitzen ditu (Ashby et al., 1990; Arvanov et al., 1999).

Bestelako erantzun batzuk, 5-$HT_{2A}$Ren bitartez ematen dira; hipertermia, hiperlokomozioa eta erantzun endokrinoak barne; hala nola kortisol, renina eta prolaktinaren jarioa areagotzea (Gudelsky et al., 1986; Barnes eta Sharp, 1999; Pytliak et al., 2011). Beste ikerketa batzuek begien kliskatzea 5-$HT_{2A}$Ren bitartez gertatzen dela frogatu dute (Welsh et al., 1998a; Welsh et al., 1998b; Romano et al., 2000; Harvey, 2003).

Aurre garunazalean espresatzen diren 5-$HT_{2A}$Rek haluzinogeno psikodelikoen erantzun psikotomimetikoekin erlazionatu dira. Normalean haluzinogenoak, LSD eta psilozibina besteak beste, erabiltzen dira eskizofreniaren sintoma positiboen antzerakoak diren sintomak eragiteko animalia ereduetan (Vollenweider et al., 1998; González-Maeso et al., 2007; González-Maeso eta Sealfon, 2009; Nichols, 2016). Egungo klinikan erabiltzen diren antipsikotiko atipikoen itu nagusia 5-$HT_{2A}$Rak dira. Klozapina, risperidona eta olanzapina, adibidez, 5-$HT_{2A}$Ren antagonista edo alderantzizko agonista gisa jokatzen dute (Meltzer, 1999).




**1.8 irudia**: 5-$HT_{2A}$R neurona piramidalen dendritetan kokatzen da. Droga psikodelikoek 5-$HT_{2A}$R aktibatzen dute, eta zelula barneko seinaleztapen bide desberdinak aktibatzen dira. Ondorioz, aktibitate neuronala areagotzen da, eta horrek ere zelula barneko bestelako seinaleztapen bideak aktibatzen ditu. Ilustrazioa: *McClure-Begley eta Roth, 2022.*

### 1.3.3 5-$HT_{2A}$Ren egitura biologikoa

Azken hamarkadan, GPCR mota desberdinen egitura biologikoak zehaztu dira, farmakologia profil desberdineko ligandoekin konplexuak sortzen dituztenak. Horrela, ligando-hartzaileen arteko eredu farmakoforoak garatu dira, eta ondorioz, mintz zeharreko hartzaileen konformazio aldaketak deskribatu dira, egitura aktiboa eratuz eta seinaleztapen bide desberdinetan eraginez (Seyedabadi et al., 2022). Homologia-ereduek eta kristalezko egiturek, agonista eta antagonista desberdinen akoplamendu-puntuak zehazteko aukera eman dute (Mozumder et al., 2020). Hartzaileen aminoazido bakarreko mutagenesiaren bidez eta ondorengo esperimentu funtzionalei esker, G-proteinen eta GPCRen artean ematen den loturan parte hartzen

duten aminoazidoak determinatzea posiblea da. 2018an Kimura eta kideek giza 5-$HT_{2A}$Ren egitura deskribatu zuten konplexuak eratuz, bigarren belaunaldiko antipsikotikoak diren risperidona eta zotepina farmakoekin lotzean (Kimura et al., 2019). Risperidona eta zotepinak hartzailearen egoera inaktiboa egonkortzen dute, eta zuzenean harremanak eratzen dituzte finkapen poltsikoaren behealdean.

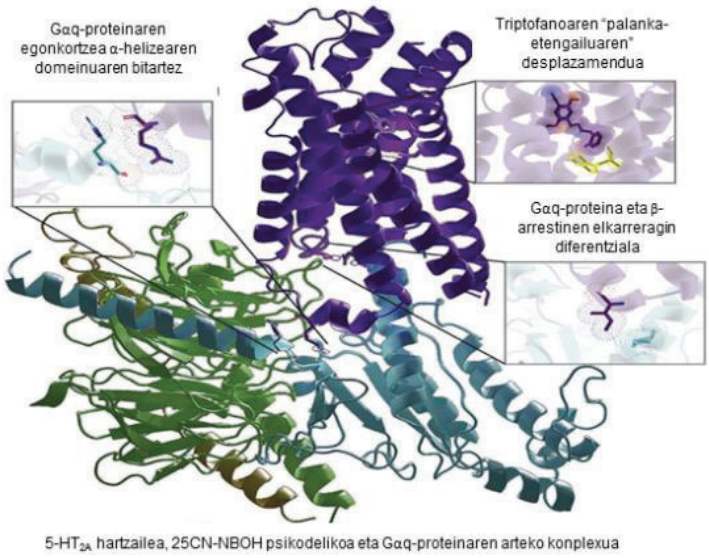
Hartzaile aminergikoen artean, 5-$HT_{2A}$Ren finkapen poltsikoaren egitura 5-$HT_{2C}$Ren antzekoa da, alderantziko agonista den ritanserinarekin konplexua eratzen duenean (Peng et al., 2018). 5-$HT_{2A}$Ren egitura 5-$HT_{2B}$R egituraren antzerakoa da baita ere, ergotamina agonistarekin konplexua eratzen duenean (Wacker et al., 2013). Horren harira, 5-$HT_{2A}$Retan risperidona eta zoteapina elkartzeko eremuak, ritanserina, risperidona eta doxepina 5-$HT_{2C}$R, $D_2$R eta $H_1$Rekin elkartzen diren guneekin gainjartzen dira, hurrenez hurren.

GPCRen egoera aktibo zein egitura ez aktiboen kristalezko egiturak lortuak dira, aldaketa konformazionalak orokorrean oso antzerakoak direlako, bereziki eremu zitoplasmatikoan. GPCRek jasaten dituzten konformazio aldaketen artean, mintz zeharreko helizeen berrantolamenduak daude, bereziki 5, 6 eta 7 helizeek jasaten dituztenak, eta badirudi zeregin kritikoa dutela seinalea mintzean zehar transmititzerako orduan (Mitra et al., 2021). Hala ere, 5-$HT_{2A}$Ren ezaugarri espezifikoak oraindik ebaluazio-fasean daude. Izan ere, aminoazido kritikoen eta farmako hautakorren arteko elkarrekintzaren heterogenotasuna handia dela deskribatu da (Wacker et al., 2013; Wacker et al., 2017; Peng et al., 2018; Kim et al., 2020). Hala, berriki frogatu da 5-$HT_{2A}$Rak LSD eta lisuriderekin eratutako konplexuen egiturak ez datozela bat 5-HT eta psilozibina konplexuekin (Cao et al., 2022). Ikerketak frogatu duenez, LSD eta lisuride egitura kimikoen zati ergolinikoa finkapen ortosteriko gunean finkatzen da, bestelako ligandoen antzera. Aldiz, 5-HT eta psilozibinaren indol egitura kimikoa finkapen ortosteriko eremuaren gainaldean kokatzen da, eta LSD eta lisurideren dietil egiturarekin lotzen dena. Laburbilduz, ikerketa horrek

5-HT eta psilozibina lotzeko bigarren modu bat erakusten du, ligando hautakor funtzionalen diseinua ahalbidetzen duena (Cao et al., 2022).

Mikroskopia elektronikoaren bidez zenbait medikamenturi lotutako hartzaileen kristal-egitura zehazteak ere ahalbidetzen du hautakortasun funtzionala edo agonismo alboratuaren mekanismoa ulertzea (Che et al., 2021; Seyedabadi et al., 2022). Horrela, 5-$HT_{2A}$R-Gαq-proteina arteko finkapen mekanismoak argitaratu ziren 5-$HT_{2A}$R eta N-(2-hidroxibenzil)-2,5-dimetoxi-4-zianopeniletil-amine (25-CN-NBOH)ren artean, farmakoa eta diseinaturiko G-proteina heterotrimerikoaren arteko konplexua aztertzean **(1.9 irudia).** Gainera, finkapen mekanismoak β-arrestinekiko hautakortasun funtzionala ere deskribatu zen, LSD farmakoa eta metiotepina (alderantzizko agonista) 5-$HT_{2A}$-R-ri lotzen zaizkionean (Kim et al., 2020).

Oro har, egituran oinarritutako agonista alboratuak diren farmakoen diseinua, farmakoen garapen prozesurako iraultzailea izango da, eta, horri esker, efektu kliniko hobetuak dituzten farmako hautakorren diseinu arrazionala egin daiteke.



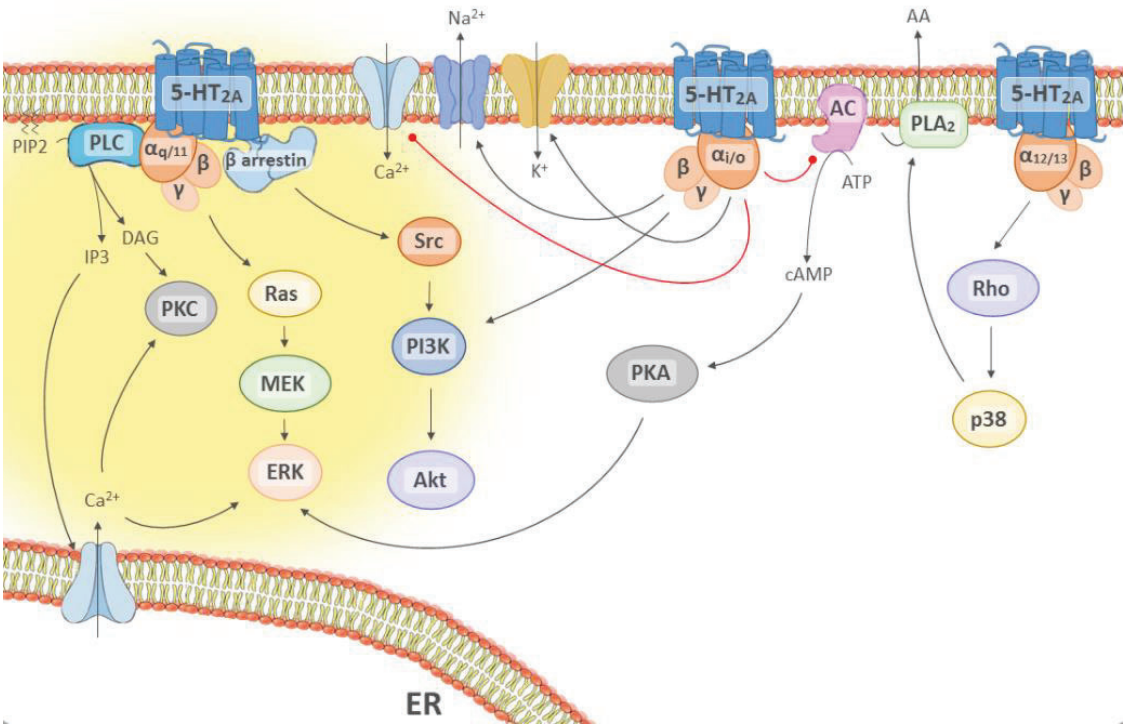

**1.9 irudia:** Mikroskopia elektroniko kriogenikoaren (Cryo-EM) bidez lorturiko 5-$HT_{2A}$R-Gαq-protein egituraren irudikapena, 25CN-NBOH farmakoari loturik. Ilustrazioa: *Kim et al., 2020.*

### 1.3.4 5-$HT_{2A}$Ren seinalestapen bideak

5-$HT_{2A}$R $G\alpha_{q/11}$-proteinetara akoplatzen da, baina bestelako seinaleztapen bideekin elkarreragin dezake, Gα-proteina azpimota desberdinak eta β-arrestinen erreklutamendua estimulatuz, besteak beste (**1.10 irudia**). Gehien ikasi den, eta ziurrekin seinaleztapen biderik garrantzitsuena dena, $G\alpha_{q/11}$-proteinak aktibaturiko PLC bidezko katalisia da, eta horren ondorioz ematen den $PIP_2$ hidrolisiarekin erlazionaturik dago (Roth et al., 1984; Hoyer et al., 1994). Aktibazio honen ondorioz, $IP_3$ sortzen da eta horren ondorioz $Ca^{2+}$ intrazularra handitzen da, eta DAG isoformak PKC eta kaltzio/kalmodulinaren mendeko kinasa II (CaMKII) aktibatzen ditu (Roth et al., 1986). Orduan, aktibatutako PKCak 5-$HT_{2A}$Ren desentsibilizazioa eragiten du, eta bestelako seinaleztapen bideen aktibazioa; mitogenoek aktibatutako proteina (MAP) kinasa (MAPK), adibidez (Banerjee eta Vaidya, 2020).

5-$HT_{2A}$Rek azido arakidonikoaren askapena (AA) sustatzen dute, $A_2$ fosfolipasa ($PLA_2$) aktibatuz (Berg et al., 1998, Parrish eta Nichols, 2006). AAren askapena oso mekanismo konplexuaren bitartez erregulatzen dela dirudi, Rhoa, MAPK eta zelulaz kanpoko seinaleek erregulaturiko kinasen (ERK) bitartez, besteak beste. Aurreko mekanismoak Gα-proteinen menpekoak dira, baina ez $G\alpha_{q/11}$-proteinaren menpekoak, $G\alpha_{12/13}$- eta $G\alpha_{i/o}$-proteina azpimoten menpekoak baizik (Kurrasch-Orbaugh et al., 2003a; Kurrasch-Orbaugh et al., 2003b). Zenbait ikerketak frogatu zuten 5-$HT_{2A}$Rek $G\alpha_{i/o}$-proteinen aktibazioa eragiteko gaitasuna dutela. Gainera, $G\alpha_{i/o}$-proteinen aktibazioa 5-$HT_{2A}$Ren bitartez gertatzen dela dirudi, droga psikodelikoek eragindako ekintza haluzinogenoen ekintza mekanismoaren arduradunak izanik (González-Maeso et al., 2007).

Gainera, ikerketa fosfoproteomikoen bidez identifikatu dira 5-$HT_{2A}$Ren aktibazioaren ondorioz, fosfoforilatzen diren zelula barneko hainbat proteina (Karaki et al., 2014). Horien artean aurkitzen dira ERK, S6 kinasa erribosomala (RSK-2) (Strachan et al., 2008) eta arrestinak (Schmid et al., 2008; Schmid eta Bohn, 2010). Gainera, 5-$HT_{2A}$Rek lotura funtzioa duten proteina askorekin elkarreragiten dutela frogatu da; hala nola 95 dentsitate proteina postsinaptikoa (PSD-95) eta PSD-95/disko luzeak/zonula-occludens 1 (PDZ)-dominioa duten proteinak (Xia Gray et al., 2003; Bécamel et al., 2004; Abbas et al., 2009), caveolin-1 (Bhatnagar et al., 2004; Sommer et al., 2009) eta mikrotubuluetara loturiko A1proteina (MAP1A) (Sheffler et al., 2006). Zehazki, deskribatu da 5-$HT_{2A}$R-ren seinaleztapen funtzionala modulatzeko elkarreragina funtsezkoa dela PSD-95, caveolina-1, RSK-2 eta β-arrestina-2 proteinekin.




**Irudia 1.10:** 5-HT$_{2A}$Ren seinaleztapen bide nagusien ilustrazioa. 5-HT$_{2A}$Ren aktibazioak G$\alpha_{q/11}$-proteinen estimulazioa ekar dezake, PIP$_2$tik IP$_3$ra bitarteko PLC bidezko katalisia eta DAGren hidrolisia sustatzen dituena, PCK aktibatuz eta $Ca^{2+}$ zitosoliko mailak handituz. Hala ere, 5-HT$_{2A}$Rak bestelako seinaleztapen bideak ere aktibatzen ditu. Adibidez, AAren askapena deskribatu da, PLA$_2$ aktibazioaren ondorioz, Rho eta p38 inplikatzen dituen mekanismo konplexu baten bitartez. Mekanismo horiek guztiak Gα-proteinen menpekoak dira, G$\alpha_{12/23}$-proteinak bezala, eta G$\alpha_{q/11}$-proteinen independenteak direnak. Iradokitzen da 5-HTak 5-HT$_{2A}$Rak aktibatzen dituela eta β-arrestinen, Src eta Aktren elkartzea eragiten duela. Aitzitik, 5-HT$_{2A}$Ren agonista haluzinogenoek gene-erregulazio diferentziala eragiten dute G$\alpha_{i/o}$ proteina azpimoten aktibazioaren bitartez. Ilustrazioa: *Ibarra-Lecue et al., 2021.*

## 1.3.5 5-HT$_{2A}$Ren ligandoak

5-HT$_{2A}$Ren finkapen farmakoak egitura kimiko desberdina duten hainbat azpitalde sailkatzen dira: indolalkilaminak, fenilalkilaminak, arilpiperazinak, alkilpiperidinak, alkilpiperazinak eta agente polizikliko/triziklikoak, besteak beste (Westkaermper eta Glennon, 2002). 5-HT$_{2A}$Retara lotzen diren

farmakoak (agonistak, antagonistak edo alderantzizko agonistak) psikiatriaren farmakoen artean garrantzitsuenen artean daude; antipsikotikoak, psikodelikoak eta antidepresiboak, besteak beste (Barnes et al., 2021). Hori dela eta, ahalegin handia egin da, 5-$HT_{2A}$Ren ligando hautakorrak garatzeko. Hala eta guztiz ere, 5-$HT_{2A}$Rekiko afinitatea duten farmako asko, 5-$HT_{2C}$Reri ere lotzen zaizkie, antzeko afinitatearekin, bi hartzaileen sekuentzia-homologia antzekoa delako.

### 1.3.5.1 Agonistak

5-$HT_{2A}$Ren farmako agonistak edo agonista partzialak betidanik 3 talde estruktural desberdinetan banatu dira: ergolinak (LSD, lisuride eta pergolide), indolalkilaminak (psilozibina) (biak indolaminen taldean) eta fenilalkilaminak (meskalina, 2,5-dimetoxi-4-iodoanfetamina ((±)DOI)) (Halberstandt eta Geyer, 2011; Nichols, 2016). 5-HT hartzaile serotonergiko guztietara lotzeko ahalmena duen agonista ez hautakorra da, eta,5-$HT_2$ hartzaileen familian, 5-HTren afinitate ordena 5-$HT_{2B}$R > 5-$HT_{2A}$R > 5-$HT_{2C}$R da (Baxter et al., 1995).

Esan bezala, 5-$HT_{2A}$Ren agonista batzuk gizakiengan efektu haluzinogenoak eragiten dituzte (LSD, psilozibina, psilozina edo meskalina). Zentzu horretan, haluzinogeno klasikoak eta psikodeliko serotonergikoak 5-$HT_{2A}$Rak aktibatuz pentsamendua, pertzepzioa eta gogo aldartea aldatzeko gai diren substantziak dira (Glennon et al., 1984, Vollenweider et al.,1998; González-Maeso et al., 2007; Halberstadt, 2015; Madsen et al., 2019). Nahiz eta eragin horiek 5-$HT_{2A}$Ren medioz gertatzen diren, haluzinogeno klasikoak ez dira farmako hautakorrak, eta bestelako hartzaileetara ere lotzen dira.

Hori horrela, fenilalkilamina taldeko haluzinogenoak 5-$HT_2$ hartzaileekio hautakorrak dira; hots 5$HT_{2A}$R, 5$HT_{2B}$R eta 5-$HT_{2C}$R. Molekula desberdinen artean, (±)DOI agonista potentea eta ez-hautakora (5$HT_{2A}$R eta 5-$HT_{2C}$Ren artean) da. Gehienetan, (±)DOI, 5-$HT_{2A}$Ren funtzioak aztertzen dituzten *in*

*vivo/ex vivo* ikerketetarako agonista eredu gisa erabili da (Nelson et al., 1999; Pigott et al., 2012; Canal et al., 2013). [$^{35}$S]GTPγS finkapen teknikaren bitartez, giza garun post-mortem eta 5-HT$_{2A}$R *kock-out* animalietan egindako esperimentuetan egiaztatu zen (±)DOI farmakoak 5-HT$_{2A}$R eta 5-HT$_{2C}$Ren agonista partzial gisa jokatzen duela (Diez-Alarcia et al., 2019; Garcia-Bea et al., 2019; Muneta-Arrate et al., 2020).

Azken urteetan zehar, 5-HT$_{2A}$Rekiko afinitate altuagoa duten farmako mota berria garatu da, N-bentzilfenetilamina (NBOMe)ren egituran oinarrituta; hala nola n-(2-metoxibentzil-2,5-dimetoxi-4-bromofeniletilamina (25B-NBOMe, Cimbi-36) eta N-(2-hidroxibentxil)-2,5-dimetoxi-4-zianofeniletilamina (25-CN-NBOH) (Jensen et al., 2020). Molekula berri horiek tresna egokiak dira PET irudi bidezko teknikan erabilitako zein azterketa farmakologikoak egiteko. 25-CN-NBOH eta Cimbi-36ak agonista partzial gisa jarduten dute, 5-HT$_{2A}$Rekiko afinitate handiagoa erakutsiz, 5-HT$_{2C}$R eta 5-HT$_{2B}$Ren aldean (Hansen et al., 2014; Jensen et al., 2017).

Bestalde, psilozina (psilozibinaren metabolito aktiboa) eta ergolinak (LSD, adibidez) hartzaile serotonergikoen agonista ez-hautakorak dira, 5-HT$_1$ eta 5-HT$_2$ hartzaileekiko afinitatea dutenak (Roth, 2007; Nichols, 2016; Wacker et al., 2017). Gainera, LSD farmakoa bestelako hartzaile serotonergiko zein hartzaile dopaminergikoetara lotzen da afinitate altuarekin (Halbertadt eta Geyer, 2011; Borroto-Escuela et al., 2014).

*Head-twitch* erantzuna (alde batetik bestera ematen den buruaren mugimendu azkarra) psikodelikoen erantzun haluzinogenoa neurtzeko gehien erabiltzen den ebaluazio froga da karraskarietan. Horregatik, *head-twich* erantzuna psikodelikoen ekintza *in vivo* ebaluatzeko froga funtzional gisa sailkatzen da. Antagonista hautakorrak eta 5-HT$_{2A}$R *knock-out* animaliak erabiliz frogatu da *head-twich* erantzuna 5-HT$_{2A}$Ren bitartez ematen dela, eta psikodelikoetara mugaturik dagoela (González-Maeso et al., 2007, Canal eta Morgan, 2012).

Hala ere, kimikoki oso antzerakoak diren molekulek, (lisuride, ergotamina eta pergolide, besteak beste), ez dute *head-twitch* erantzuna eragiten, nahiz eta 5-$HT_{2A}$Rekiko afinitate altua izan (González-Maeso et al., 2003; González-Maeso et al., 2007). Hori dela eta, lisuride eta pergolide 5-$HT_{2A}$Ren agonista ez-aluzinogeno gisa sailkatzen dira. Molekula horiek, bestelako ergolinak bezala, profil farmakologiko oso konplexua dute, eta hainbat hartzaile aminergikoekiko kidetasuna aurkezten dute, hartzaile serotonergikoak eta dopaminergikoak barne (Halberstadt eta Geyer, 2011). Gainera, lisuride eta pergolide, parkinson gaixotasunaren aurkako farmako gisa erabiltzen dira, dopamina eta 5-$HT_{1A}$ hartzaileekiko duten afinitate altua dela-eta (Langtry eta Clissold, 1990; Marona-Lewicka et al., 2002).

### 1.3.5.2 Antagonistak

N-alkilpiperidonak 5-$HT_{2A}$Ren taldeko antagonista klase handiena eta hautakorrenen artean dago. Horietatik, ketanserina izan da urteetan zehar antagonista gisa erabili izan den farmako nagusia. Ketanserinak hautakortasun altuagoa dauka 5-$HT_{2A}$Rekiko 5-$HT_{2C}$Rekiko baino (15-80 aldiz), eta baita 5-$HT_{2B}$Rekin alderatuz (500-1000 aldiz) (Jerman et al., 2001; Knight et al., 2004; Diez-Alarcia et al 2019). Kimikoki, ritanserina eta ketanserina oso antzekoak dira, 5-$HT_{2A}$Ren antagonista potente eta hautakor zein alderantzizko agonista gisa deskribatu direnak biak (Bonhaus et al., 1995). Beste 5-$HT_{2A}$R antagonista, kimikoki ketanserinarekin erlazionatua ere, altanserina da; 5-$HT_{2A}$Ren antagonista potente eta hautakor gisa deskribatu dena, 5-$HT_{2A}$Rekin 20 aldiz afinitate altuagorekin lotzen dena bestelako hartzaileekin baino (Tan et al., 1999). Hala ere, alderantzizko agonista ezaugarriak ere deskribatu zaizkio altanserinari (Aloyo et al., 2009; Diez-Alarcia et al., 2019).

5-$HT_{2A}$Ren beste ligando hautakor batzuk ere garatu dira; hala nola MDL100907, bolinanserina ere deiturikoa, eta MDL-11,939. Bolinanserina 5-$HT_{2A}$Ren antagonista potentea da, eta 300 aldiz afinitate altuagoa du 5-$HT_{2A}$Rekiko, 5-$HT_{2c}$Rekiko baino, eta baita beste hartzaileekin konparatuz (Sorensen et al., 1993; López-Giménez et al., 1998). Bolinanserina eta ritanserina antipsikotiko gisa ebaluatu ziren eskizofreniaren tratamendurako, hala ere, ez zuten arrakastarik izan, eta ondorioz beren erabilera mugatu egin zen (Jones et al., 2020). Gaur egun, bolinanserina 5-$HT_{2A}$Ren antagonista eredu gisa erabiltzen da, hautakortasun handia duelako.

Duela gutxi, farmako berriak garatu dira loezina tratatzeko, nelotanserina eta eplibanserina, besteak beste. Konposatu berri horiek 5-$HT_{2A}$Rekiko afinitate altua aurkezten dute, 20 aldiz hautakortasun gehiago 5-$HT_{2A}$Rekiko baino (Rinaldi-Carmona et al., 1992; Al-Shamma et al., 2010).

Pimabanserina, berriz, ACP-103 bezala ere ezagutzen dena, 5-$HT_{2A}$Ren farmako oso hautakora da, beste hartzaileekiko afinitaterik ez duena, eta 5-$HT_{2A}$Rekiko 30 aldiz afinitate altuagoa aurkezten duena 5-$HT_{2C}$Rekiko baino(Vanover et al., 2006; Abbas eta Roth, 2008). Pimabanserina Ameriketatako Estatu Batuetako Drogen eta Elikagaien Administrazioak (FDA) farmako gisa onartu du Parkinson gaixotasunean gertatzen den psikosiarekin lotutako haluzinazioak eta eldarnioak tratatzeko (Cummings et al., 2014).

5-$HT_{2A}$Ren beste antagonista batzuk, nahiz eta 5-$HT_{2A/2C}$Rekiko hautakorrak izan, hartzaile dopaminergiko, histaminergiko edo/eta adrenergikoekiko afinitate ertain eta altuarekin lotzen dira. Horietaz gain, antipsikotiko atipikoak (adibidez, risperidona, klozapina eta olanzapina) eta antidepresibo triziklikoak (adibidez, amitriptilina, klomipramina eta imipramina) ere 5-$HT_{2A}$Retara lotzen dira antagonista gisa (Roth et al., 2004; Meltzer eta Massey, 2011; Meltzer, 2012).

### 1.3.6 5-$HT_{2A}$Ren hautakortasun funtzionala

Hartzaile serotonergikoak, 5-$HT_{2A}$R bereziki, hautakortasun funtzionala zuela iradoki zen lehen GPCRetakoa izan zen (Berg et al., 1998). Zenbait ikerketen arabera, 5-$HT_{2A}$Ren agonista zein antagonistek, hartzailearen konformazio desberdinak egonkortzen dituzte. Horren ondorioz, hautakortasun funtzionala sortzen da seinaleztapen bide desberdinen bidez; $G\alpha_{q/11}$-proteinen seinaleztapen bideaz gain bestelako seinaleztapen bide alternatiboak aktibatuz (López-Giménez eta González-Maeso, 2018). Ildo horretan, hautakortasun funtzionala proposatu da erantzun zelular desberdina eragitearen fenomenoa azaltzeko, 5-$HT_{2A}$R kortikalen populazio bera aktibatzen baitute droga haluzinogenoek, eta ez-haluzinogenoak diren farmakoek. Baina erantzunen artean desberdintasunak aztertu dira Gα-proteinetan, geneen espresioan, erantzun elektrofisiologikoan eta portaera probetan (González-Maeso et al., 2003; González-Maeso et al., 2007; Karaki et al., 2014; Banerjee eta Vaidya, 2020).

Hautakortasun funtzionalaren lehen ebidentzietakoa Berg eta kolaboratzaileen aurkikuntzatik sortu zen; hartzaileek $G\alpha_{q/11}$-proteinen mendeko PLCaren aktibazioaz gain, $PLA_2$ren aktibazioa ere gertatzen zela frogatu zutenean (Berg et al., 1998). Zehazki, PLC mendeko IP mailak eta $PLA_2$ren aktibazioaren ondoriozko AAren askapena neurtu zuten, seinaleztapen bide bakoitza aktibatzeko eraginkortasun ahalmena neurtzeko. Emaitza horien arabera, 5-HTak PLC-IP seinaleztapen bidea aktibatzen zuen bereziki, eta LSDak, berriz, $PLA_2$-AA bidea (Berg et al., 1998; Martí-Solano et al., 2015).

Gainera, 5-$HT_{2A}$R haluzinogeno eta ez-haluzionogenoek eragin diferentziala dute gene-adierazpenean (González-Maeso et al., 2003; González-Maeso et al., 2007). 5-$HT_{2A}$R agonista haluzinogeno eta ez-haluzinogenoen presentzian, gene erantzun desberdina ematen dela frogatu zuten zelula eta saguen garunazal somatosentsorialean. Hori horrela, droga haluzinogenoak

zein ez-haluzinogenoak c-fos genearen adierazpena eragiten dute. Aldiz, erg-1 eta erg-2 geneen adierazpena soilik gertatzen da LSD eta (±)DOI agonista haluzinogenoen presentzian, baina azken bi geneen adierazpena ez da aldatzen lisuride eta ergotamina agonista ez-haluzinogenoen presentzian (**1.11 irudia**). Emaitza horien arabera, 5-$HT_{2A}$Ren agonista guztiek PLCrekin akoplaturiko 5-$HT_{2A}$R aktibatzen dituzte. Haluzinogenoen mendeko erantzunak, aldiz, PTX sentikor diren $G\alpha_{i/o}$-proteinek bideratzen dituzte. Ikuspegi berri hori, fosfoproteomika kuantitatiboan oinarritaturiko esperimentuen bitartez balioztatu zen (Karaki et al., 2014). Gainera, (±)DOI agonista haluzinogenoak eta pergolide agonista ez-haluzinogenoak hautakortasun funtzional desberdina aurkeztu zuten post-mortem giza aurre-garunazalean (Muneta-Arrate et al., 2020). Bi agonistek $G\alpha_{q/11}$-proteinen aktibazioa eragiten dute; $G\alpha_{i1}$-proteinaren aktibazioa, berriz, (±)DOI droga haluzinogenoak soilik eragiten du (Muneta-Arrate et al., 2020).

Ondorioz, agonista haluzinogenoek, LSD eta psilozibina kasu, $G\alpha_{q/11}$-proteinak zein $G\alpha_{i/o}$-proteinak aktibatzen dituzte. Aitzitik, lisuride, ergotamina eta pergolide, 5-$HT_{2A}$Ren agonistak izanik, nahiz eta egitura kimiko antzekoa izan, ez dituzte ezaugarri haluzinogenoak, $G\alpha_{q/11}$-proteinen mendeko bidea baino ez dutelako estimulatzen.

Beste ikerketa batek, 5-$HT_{2A}$Ren agonista haluzinogenoak eta ez-haluzinogenoak seinaleztapen sinadura desberdina zutela frogatu zuen. Egileek, fosforilaturiko-PLC, pERK, pCREBII zein IP eta DAG ekoizpen maila altuagoak neurtu zituzten (±)DOIren presentzian lisuride farmakoarekin konparatuz (Banerjee eta Vaidya, 2020).

Gainera, 5-$HT_{2A}$Rek G-proteinekiko independenteak diren beste seinaleztapen bideak aktibatzen dituzte, β-arrestinak besteak beste. 5-HTak eta (±)DOIk modu bereizgarrian aktibatu ditzakete 5-$HT_{2A}$Rak eredu zelularretan eta *head-twich* erantzuna neurtzean. β-arrestina-2 proteinarik

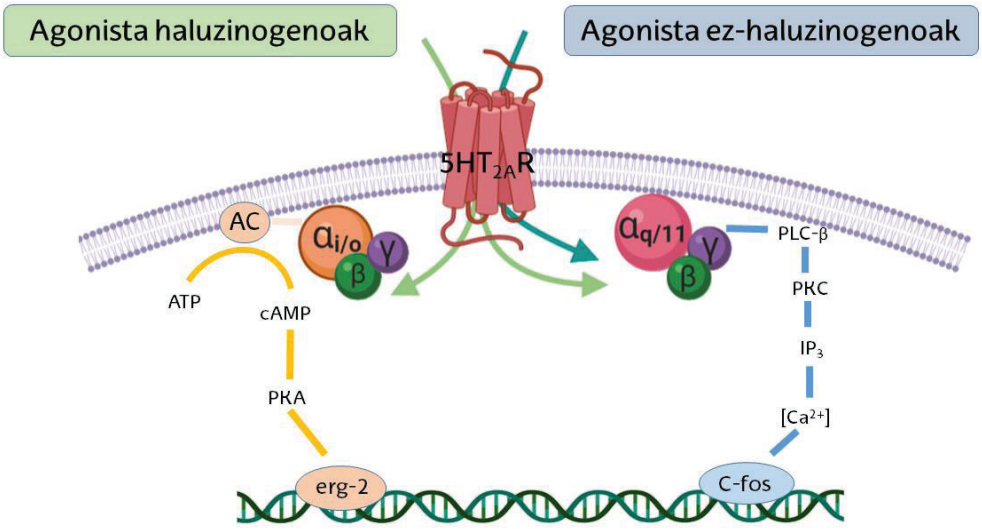
gabeko animalietan, 5-HTak *head-twitch* erantzuna sortzeko ahalmena galtzen duela frogatu zuten. Badirudi, Akt-ren fosforilazioa gertatzen dela β-arrestina-2ren aktibazioaren ondoren, baina animalia horietan (±)DOI farmakoaren aktibazioaren ondoren ez da Akt-ren aktibaziorik gertatzen. Hala eta guztiz ere, (±)DOI farmakoak eragindako *head-twich* erantzuna β-arrestina-2arekiko independentea dela dirudi. Egitura kimiko desberdina duten agonistek 5-$HT_{2A}$R aktibatu ondoren seinaleztapen bide desberdinak aktibatzeko ahalmena dutela ondorioztatu zen (Schmid et al., 2008; Schmid eta Bohn, 2010).

Berriki iradoki zen LSD droga haluzinogenoak eragiten zuen *head-twich* erantzuna ere β-arrestina-2-mendekoa zela, baina β-arrestina-1rekiko independentea (Rodriguiz et al., 2021). Hala ere, beste ikerketa batean frogatu zuten agonista ez haluzinogenoek ere β-arrestina-2 erakartzeko ahalmena zutela, aurreko aurkikuntzaren emaitzen kontra (Cao et al., 2022). Beraz, β-arrestina-2 eta haluzinogenoen ekintza mekanismoak ikerketa sakonagoa behar du.

Bestalde, klozapina antipsikotiko atipikoak 5-$HT_{2A}$Ren seinaleztapena blokeatzeko ahalmena du, G-proteinen bitartez. Hartzailea zelulan barneratzea (internalizazioa) eragiten du, eta baita Akt-ren fosforilazioa, nahiz eta β-arrestina-2rekin ez elkarreragin. Beraz, 5-HT eta klozapinak mekanismo desberdina erabiltzen dute, 5-$HT_{2A}$Ren bitartez, seinaleztapen bide berdinak induzitzeko: Akt-ren fosforilazioa eta hartzailearen internalizazioa. Hori horrela, klozapinak Akt fosforilazioaren bitartez blokeatzen du 5-$HT_{2A}$R, eta PCP edo MK-801 farmakoek eragindako sintoma psikotikoak ere blokeatzeko gai da.

Hautakortasun funtzionalaren konplexutasuna bide alternatiboa izan daiteke farmako hautakorren garapenean, eragin kliniko areagotuak eta eragin desiragaitz gutxituak dituzten farmakoak garatzeko orduan. Hala ere,

hautakortasun funtzionala eta bestelako ezaugarri farmakologikoak, hala nola alderantzizko agonismoa edo antagonismoa, farmako berrien mekanismo farmakologiko gisa zalantzan jarriak dira oraindik, depresioa eta eskizofrenia gaixotasunen terapia gisa.




**1.11 Irudia**: 5-$HT_{2A}$Ren seinaleztapen intrazelularraren irudikapen eskematikoa

## 1.4 5-$HT_{2A}$Rak eta eskizofrenia

Hainbat ikerlanetan aztertu dute 5-$HT_{2A}$Ren eta eskizofreniaren arteko harremana. Eskizofrenian 5-HT neurotransmisorearen inplikazioa zehaztea zaila izan bada ere (Halberstadt & Geyer, 2013), ikerketa gehienek 5-$HT_{2A}$R aztertu dute antipsikotiko atipikoek 5-$HT_{2A}$R blokeatzeko ahalmenean oinarriturik (Meltzer et al., 1989; Miyamoto et al., 2005). Gainera, lehen adierazitakoari jarraituz, hainbat drogek, sintoma psikotikoak eragiten dituzte 5-$HT_{2A}$R-en aktibazioaren ondorioz, bai gizakietan bai karraskarietan; psilozibinak, LSDak eta (±)DOIk, adibidez.

### 1.4.1 Ikerketa genetikoak

HTR2A genearen aldaera eta eskizofreniaren arteko erlazioa hainbat ikerlanek proposatu dute. Hala, HTR2A genearen edo promotorearen mutazioak eskizofreniarekin erlazionatu izan dira, nahiz eta, horietako bat bera ere ez den populazioan modu sendoan erreplikatu. Izan ere, eskizofrenian egindako GWAS azterketek ez zuten inolako aldaketa esanguratsurik aurkitu HTR2A gene aldaeren artean (Farrell et al., 2015).

Gainera, eskizofrenian aztertu diren HTR2A espresioaren aldaketak nukleotido bakarreko polimorfismoekin (SNPs) ere erlazionatu izan dira: A-1438G eskualde promotorean (Ohara et 1998), His452Tyr eskualde kodifikatzailean (Ozaki et al., 1996), eta T102C lehenengo exonean (Arranz et al., 1996), besteak beste. Azterlan batzuk SPN horiek eskizofrenian parte hartzea bultzatzen duten arren, beste ikerketa batzuek kontrakoa frogatu dute.

Horien artean, zenbaitek iradoki dute A-1438G polimorfismoa eskizofrenia izateko probabilitatearekin erlazionaturik dagoela (Parsons et al., 2004; Peñas-Lledo et al., 2007; Sáiz et al., 2007; Smith et al., 2013). Era berean, frogatu da A-1438G polimorfismoak eragina duela tratamendu antipsikotikoaren erantzunean ere (Yan et al., 2021).

Azkenik, His452Tyr (rs6314) eskizofreniarekin zer ikusia duela frogatu zen, baina are gehiago antipsikotikoen erantzunarekin lotu dute. Polimorfismoa klozapina eta olanzapinaren erantzun kliniko aldakorrarekin erlazionatu da (Birkett et al., 2000; Olajossy-Hilkesberger et al., 2011). Gainera, HTR2A rs6314 $5HT_{2A}$ren adierazpen eta funtzioan eragiten du, eta horrek eskizofreniaren endofenotipoa modulatzera laguntzen du, hala nola portaera kognitiboak eta horri lotutako aktibitate prefrontala (Blasi et al., 2013).

Bestalde, T102C (rs6313) erlazionatu da populazio desberdineko eskizofrenia gaixotasunarekin, baita risperidona eta olanzapinaren antipsikotikoen erantzunarekin (Petronis et al., 2000; Maffioletti et al., 2020).

Laburbilduz, goiko datuen sendotasun ezak, 5-$HT_{2A}$Ren funtzio, espresio eta antispikotikoen erantzunean eragina duten SPNei buruzko etorkizunean egin daitezkeen ikerketa prekliniko nahiz kliniko gehigarriak egitea gehiago justifikatzen ditu.

### 1.4.2 5-$HT_{2A}$Ren dentsitatea, espresioa eta funtzionalitatea subjektu eskizofrenikoetan

Eskizofrenia duten subjektuen post-mortem garunean 5-$HT_{2A}$Ren RNAm ebaluatzean emaitza desberdinak argitaratu dira. Eskizofrenia duten subjektuen aurre-garunazalean, 5-$HT_{2A}$Ren RNAmaren adierazpenean murrizketak nahiz aldaketa ezak zituzten emaitzak argitaratu dira azken urteotan (Burnet et al., 1996; Hernandez & Sokolov, 1997; Hernandez & Sokolov, 2000; Lopez-Figueroa et al., 2004).

Duela gutxi, tratamendu antipsikotikoa jaso zuten eta tratamendurik gabeko subjektuen post-mortem aurre-garunazalean 5-$HT_{2A}$Ren RNAm adierazpena aztertu zen. Emaitzen arabera, antipsikotikorik gabeko subjektu eskizofrenikoetan, 5-$HT_{2A}$Ren RNAmren adierazpena, kontrolekin konparatuz,

antzekoa izan zen. Aldiz, antipsikotikoekin tratatutako eskizofrenikoetan RNAmren adierazpen murriztua behatu zen (Garcia-Bea et al., 2019). Beste ikerketa batean ere antzerako emaitzak lortu zituzten, aurreko emaitzak bultzatzen zituena (Zhao et al., 2022).

Eskizofrenia duten subjektuen post-mortem garun ehunean, 5-$HT_{2A}$Ren adierazpen eta dentsitateari buruzko emaitza kontrajarriak argitaratu dira. Normalean, 5-$HT_{2A}$Ren dentsitatea *in vivo* PET teknika eta *in vitro* post-mortem ehun homogenizatuak edo sekzioak erabiliz aztertzen da. Horrela, post-mortem azterketa batzuek 5-$HT_{2A}$Ren dentsitatea altuagoa zela eutsi zuten bitartean, beste batzuek ez zuten aldaketarik ikusi, edo hartzaileen dentsitatean murrizketa frogatu zuten.

Emaitzen desadostasunak nahasmendua hainbat faktoreren araberakoak dira: neurri demografikoak eta ezaugarri klinikoak, adina, antipsikotikoen edo beste psikofarmakoen tratamendua zein suizidioa besteak beste, post-mortem garun ehunean egindako azterketen kontrako emaitzen ondorio dela argudiatu zen (Dean, 2003; González-Maeso eta Sealfon, 2009). Gainera, erabilitako erradioligandoa edo laginaren prestakuntza bezalako faktore metodologikoek kontrako emaitzak ekar ditzaketela ere iradoki da, eskizofrenia duten subjektuen post-mortem garunekin erabiltzean (Dean et al., 2008; Diez-Alarcia et al., 2021a).

Izan ere, post-mortem garunean egin ziren lehenengo azterketetan LSD, 5-$HT_{2A}$Ren agonista partzial ez selektiboa, erabili zen. Emaitzen arabera, paziente eskizofrenikoetan 5-$HT_{2A}$Ren dentsitatearen murrizketa nabarmena behatu zen, garuneko eremu kortikal desberdinetan (Perotuka eta Snyder, 1979). Hala ere, badira bestelako ikerketak kontrako emaitzak argitaratu zituztenak [$^3$H]LSD erabiliz (Joyce et al., 1993; Gurevich eta Joyce, 1997). Duela gutxi, [$^3$H]LSD erradioligandoa erabiliz 5-$HT_{2A}$Ren dentsitate altuagoa ere aurkitu zen (Diez-Alarcia et al., 2021a).

Horietaz gain, [$^{18}$F]setoperona eta [$^{18}$F]-methil espiperonaren presentzian, hartzailearen dentsitate handipena, murrizketa edo inolako aldaketarik ez zegoela ere aurkitu zen (Trichard et al., 1998; Ngan et al., 2000; Verhoeff et al., 2000; Okubo et al., 2000). Nabarmentzekoa da, erradiotrazadoreek dopamina hartzaileekiko afinitate altua ere dutela, 5-HT$_{2A}$Rez gain, eskizofreniaren fisiopatologian ere parte hartzen dutenak.

Aitzitik, *in vivo* PET eta *in vitro* finkapen tekniketan [$^{18}$F]altanserina erabili zenean, 5-HT$_{2A}$Ren erradioligando selektiboa dena, kontrako emaitzak lortu ziren. Azterketa horien arabera, tratamendurik gabeko eskizofrenikoen garunazalean 5-HT$_{2A}$Ren murrizketa behatzen da (Erritzoe et al., 2008; Rasmussen et al., 2010; Rasmussen et al., 2016; Diez-Alarcia et al., 2021a).

Gainera, zenbait ikerketek post-mortem garunean 5-HT$_{2A}$Ren dentsitatea [$^{3}$H]ketanserinarekin ebaluatu dute. Hori erabiliz, tratamendu antipsikotikoa zuten eskizofrenikoen 5-HT$_{2A}$R-ren dentsitate murrizketa edo aldaketarik ez dagoela aurkitu zuten (Dean et al., 1998; Pralong et al., 2000; Dean et al., 2008). Klozapina antipsikotikoaren tratamendu kronikoaren ostean, berriz, 5-HT$_{2A}$R-en beheranzko erregulazioa deskribatu zen (Garcia-Bea et al., 2019). Alderantziz, [$^{3}$H]ketanserinarekin egindako azterketa batean, heriotzaren orduan odolean antipsikotikorik ez zituzten eskizofrenikoen garunean, 5-HT$_{2A}$Ren dentsitatearen handipena dagoela frogatu zuten (González-Maeso et al., 2008, Muguruza et al., 2013).

Azkenik, PET azterketetan [$^{11}$C]MDL100907 erradioligandoa askotan erabili da 5-HT$_{2A}$Ren antagonista selektibo gisa (L´Estrade et al., 2018). Harrigarria bada ere, *in vitro* post-mortem giza garunean egindako ikerketak ez zuen aldaketarik frogatu 5-HT$_{2A}$Ren dentsitatean (Diez-Alarcia et al., 2021a).

Ikuspuntu farmakologikotik, posible da kontrakoak diren emaitzei azalpena ematea. Aurretik aipatu den moduan, 5-HT$_{2A}$R konformazio molekular aktiboan (G-proteinetara akoplaturik) eta inaktiboan (G-proteinetara

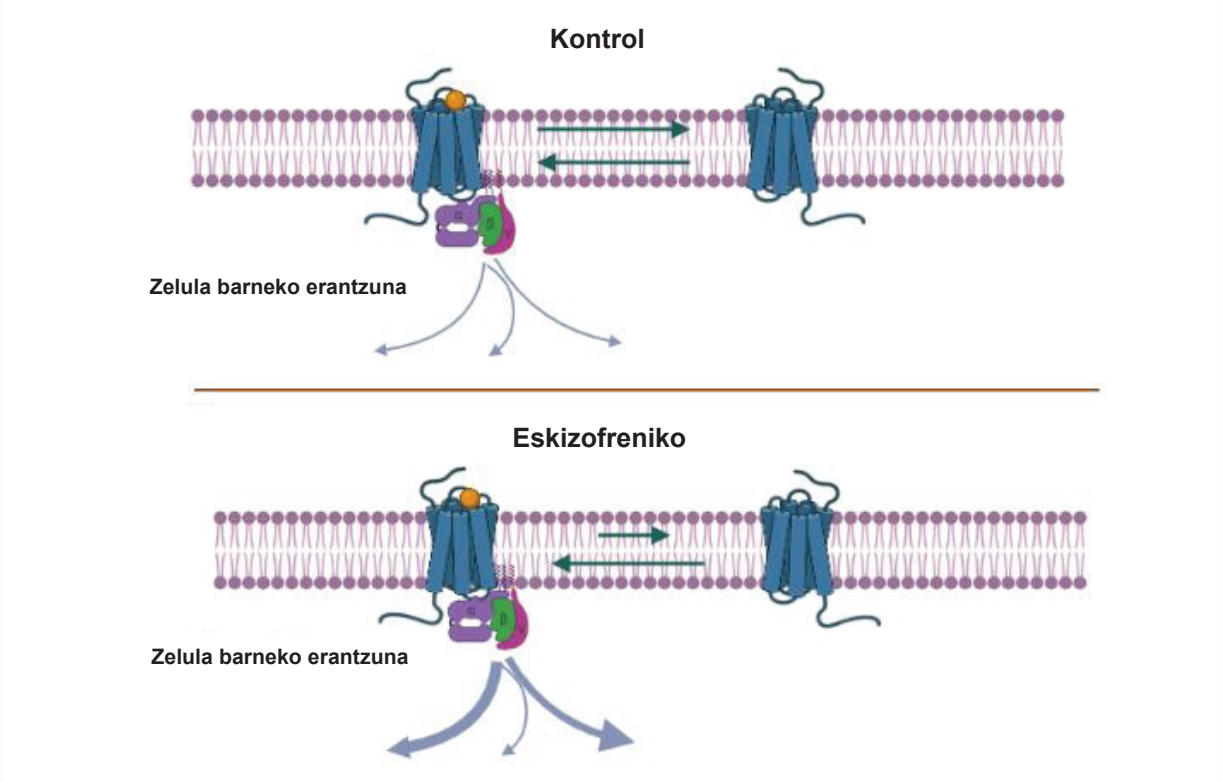
desakoplaturik) orekan aurkitzen da (Battaglia et al., 1984; López-Giménez et al., 2001) (**1.12 Irudia**). Erradiotrazadore antagonista selektiboak afinitate bera dute hartzailearen bi konformazioekiko. Aitzitik, ligando agonistak eta alderantzizko agonistek afinitate desberdina erakusten dute hartzaile populazio desberdinekiko (konformazio aktibo eta inaktiboarekiko) (Kenakin, 2011). Afinitatean aurkezten duten desberdintasunek, nolabait azaldu dezakete emaitzetan ikusitako diferentziak. Zentzu honetan, emaitzen interpretazio egoki baterako, kontuan hartu beharko lirateke erradiotrazadoreen ezaugarri farmakologikoak, eta emaitzak berriro ebaluatu.

Beraz, nahiz eta altanserina eta bolinanserina antagonista moduan deskribatu ziren, altanserina alderantzizko agonista gisa deskribatu da giza post-mortem garunean (Diez-Alarcia et al., 2019). Bestalde, ketanserina normalean antagonista gisa erabiltzen zen farmakoa izan arren, agonista partzial gisa ere deskribatu da (Muguruza et al., 2013). Beraz, altanserina afinitate baxuko konformaziora finkatuko da, eta ,aldiz, LSD eta ketanserinak, agonista ezaugarriak izanda, afinitate altuko konformazioa ezagutuko dute gehienbat.

Eskizofrenikoen post-mortem garuneko *in vitro* azterketek argia eman zuten 5-$HT_{2A}$Rei buruz (Diez-Alarcia et al., 2021a). Ikerketa horietan subjektu eskizofrenikoetan konformazio aktiboan aurkitzen diren 5-$HT_{2A}$Ren dentsitate handiagoa aurkitu zuten, erradiotrazadore agonistek detektatzen dutenarekin bat eginez. Halaber, konformazio ez-aktiboaren murrizketa ere baieztatua izan zen, batez ere alderantzizko agonisten bidez detektatzen zenean. Espero zitekeen bezala, erradiotrazadore antagonistek ez dituzte hartzaileen konformazio desberdinak bereizten, eta ,ondorioz, ez ziren aldaketarik behatu hartzaileen dentsitatean.

5-$HT_{2A}$R kortikalak eskizofrenia duten subjektuetan selektibitate funtziona aurkezten dute $G\alpha_{i1}$-proteinen bidez, eta $G\alpha_{q/11}$ seinaleztapen kanonikoaren bidez, berriz, aldaketa eza (Garcia-Bea et al., 2019; Odagaki et al., 2021).

Aurrez aipatutako erradioligandoen eta funtzionalitateari buruzko emaitzen arabera, 5-$HT_{2A}$Ren sentikortasunaren handipena ez da hartzaileen dentsitate edo sintesi handipenaren ondorioz, aurretik uste zen moduan. Izan ere, eskizofrenia duten subjektuen 5-$HT_{2A}$Ren alterazioa G-proteinei akoplatutako (konformazio aktiboa) eta ez akoplaturiko (konformazio inaktiboa) egoeren arteko konformazio-trukearen asaldurarekin loturik dagoela dirudi (Muguruza et al., 2013; Diez-Alarcia et al., 2021a).




**1.12 irudia:** 5-$HT_{2A}$Ren konformazio desorekaren irudikapen grafikoa. Subjektu eskizofrenikoetan agonistek 5-$HT_{2A}$Ren konformazioa oreka finkaturiko konformaziorantz orekatzen da gehienbat. *Diez-Alarcia et al., 2021atik* eraldatua.

## 1.5 Antipsikotikoak

Antipsikotikoak eskizofrenia tratatzeko lehen lerroko medikazio gisa erabiltzen dira, eta bi kategoria desberdinetan sailkatzen dira: lehen belaunaldiko antipsikotikoak edo antipsikotiko tipikoak (adibidez, haloperidol eta klorpromazina) eta bigarren belaunaldiko antipsikotikoak edo antipsikotiko atipikoak (adibidez, klozapina eta risperidona).

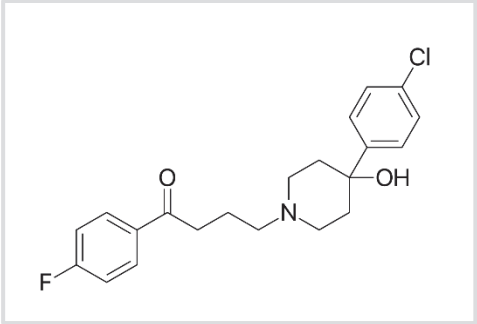
Eskizofrenia tratatzeko dauden sendagaiak sintoma positiboak tratatzeko eraginkorrak dira, baina eraginkortasun mugatua dute sintoma negatibo edo kognitiboak tratatzeko orduan (Conn et al., 2008; Leucht et al., 2009). Horrek, antispikotikoen farmakoterapiak emaitza funtzional txarrak izatera bultzatzen du. Horrenbestez, premiazkoa da itu molekular berriak identifikatzea, eta ekintza mekanismo berriak dituzten farmakoak garatzea, eragin terapeutiko eta segurtasun profila hobetu ditzaketen agente antipsikotiko eraginkorrago eta jasangarriagoak izateko.

### 1.5.1 Lehen belaunaldiko antipsikotikoak (antipsikotiko tipikoak)

Antipsikotiko tipikoen ekintza mekanismoa $D_2R$, $D_3R$ eta/edo $D_4R$en antagonismoan oinarritzen da. Ekintza mekanismo horiek ezagutzearen ondorioz eskizofrenian gertatzen den sistema mesolinbikoaren hiperdopaminergia eta hipodopaminergia kortikala deskribatzen duen “sistema dopaminergikoaren hipotesia” garatu zen. Horren arabera, $D_2R$ blokeoa dopaminaren eta sintoma psikotikoen murrizketarekin erlazionatzen da (Seeman, 1992; Marder et al., 1993). $D_2R$en blokeoa albo-ondorio estrapiramidalekin eta hiperprolaktinemiarekin ere loturik dago (Miyamoto et al., 2008).

Horren harira, haloperidola, lehen belaunaldiko antipsikotiko prototipikoa dena, aitzina oso erabilia izan da (**1.13 irudia**). Antipsikotiko atipikoek

sintomatologia positiboa murrizteko gaitasuna dute, eskizofrenia pairatzen duten pazienteen kuadro klinikoa hobetuz. Hala ere, pazienteen %30ak, gutxi gora behera, erantzun murriztua erakusten dute, edo inolako erantzunik ez antipsikotiko tipikoak ematean. Gainera, ez dute inolako onurarik eragiten sintoma negatibo edo asaldura kognitiboetan (Conley eta Kelly, 2001; Legge et al., 2020).




**1.13 irudia**: Haloperidolaren egitura kimikoa.

## 1.5.2 Bigarren belaunaldiko antipsikotikoak (antipsikotiko atipikoak)

Bigarren belaunaldiko antipsikotikoak, edo antipsikotiko atipikoak, albo-ondorio estrapiramidalak murrizteko helburuarekin garatu ziren (**1.14 irudia**). Antipsikotiko atipikoek afinitate altuagoa aurkezten dute 5-$HT_{2A}$Rekiko, $D_2$R familiarekin alderatuta. ezaugarri hori síntoma extrapiramidalen murrizketarekin erlazioanatua dago,eta ,beraz, 5-$HT_{2A}$/$D_2$ hartzaileen afinitate erlazioa eragin desigaitzen profila aurreikusteko eralbilgarria da (Ebdrup et al., 2011). Zoritzarrez, bigarren belaunaldiko antipsikotikoek pisu handitzea, eta glukosa zein lipidoen metabolismoan aldaketak izateko arriskua handitzen dute (Muench eta  Hamer, 2010; Weston-Green et al., 2013; Grajales et al., 2019).

Klozapinaren garapenak, lehen belaunaldiko farmakoekin alderatuta, perfil farmakologiko hobetua zuten farmako berrien garapenean lagundu zuen (Meltzer et al., 1989). Klozapinaren profil farmakologiko konplexua dela eta, oso zaila da farmakoaren eragin klinikoen ekintza mekanismoa zehaztea. Klozapinak hartzaile desberdinekiko afinitate esanguratsua aurkezten du; hartzaile histaminergiko, adrenergiko, dopaminergiko eta kolinergikoekiko, besteak beste (Coward, 1992; Nucifora et al., 2017). $D_2R$ eta 5-$HT_{2A}$Retan eragiteaz gain, klozapina 5-$HT_1R$ agonista partziala ere bada, sintoma kognitibo eta negatiboen murrizketan inplikaturik daudenak. Hartzaile muskarinikoetan ere eragiten du; $M_1R$, $M_2R$, $M_3R$ eta $M_5R$ blokeatuz, $M_4R$ estimulatzen duen bitartean. Gainera, hartzaile histaminergikoak blokeatuz sedazio efektua sortzen du. Azkenik, klozapina hartzaile adrenergikoak blokeatzen ditu, hipotentsioa eta takikardia eraginez (Coward et al., 1992; Nucifora et al., 2017).

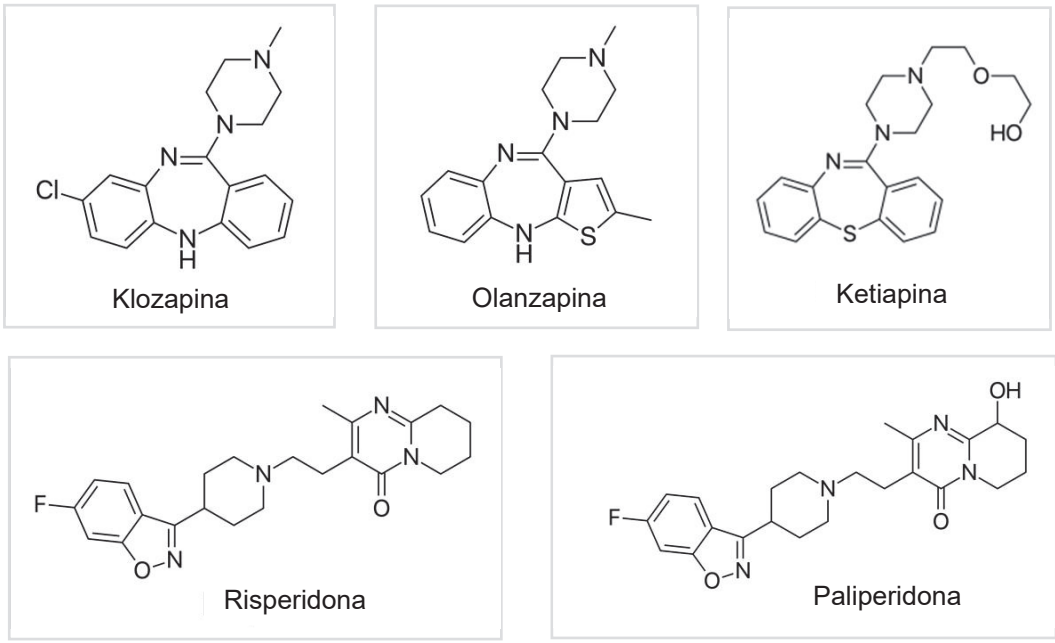
Klozapina farmakoa hilgarria izan daiteke, hemototoxikotasun-arriskuaren (agranulozitosia eta neutropenia) ondorioz. Hori dela eta, bere erabilera klinikoa mugaturik aurkitzen da (Alphs et al., 1991). Hori horrela, bigarren belaunaldiko antipsikotiko berriak garatu ziren; hala nola risperidona, olanzapina eta ketiapina, odol-diskrasiekin loturiko albo-ondorioak murrizteko helburuarekin.

Olanzapina klozapinaren analogo kimikoa da, antzeko ezaugarri farmakologikoak dituena, baina agranulozitosi arriskurik gabe. Espero zen moduan, olanzapinak 5-$HT_{2A}$Rekiko afinitate handiagoa du hartzaile dopaminergikoekiko baino. Hartzaile histaminergikoak, muskarinikoak eta adrenergikoak blokeatzen ditu, baina potentzia gutxiagorekin, klozapinren aldean. Hala ere, pisu handitzea eta sedazioa bezalako eragin desiragaitzak sortzen ditu olanzapinak (Fulton eta Goa, 1997; Leucht et al., 2013).

Ketiapinak, berriz, $D_1$R, $D_2$R eta 5-$HT_{2A}$Ren antagonista zein 5-$HT_{1A}$Ren agonista partzial gisa jarduten du. Ketapinak eragindako albo-ondoriok antagonismo $\alpha_1$-adrenergiko eta histaminergikoarekin erlazionaturik daude (Miodownik eta Lerner, 2006).

Risperidona ere antipsikotiko atipikoa da. Risperidonaren eragin terapeutikoa 5-$HT_{2A}$R eta $D_2$Ren blokeoan oinarritzen da, baina 5-$HT_{2A}$Rekiko afinitate handiagorekin (Cohen, 1994). Horrez gain, risperidona $\alpha_1$-adrenohartzaileak eta hartzaile histaminergikoak blokeatzen ditu. Risperidona sintoma positiboen tratamendurako eraginkorra izateaz gain, nahasmen kognitibo eta sintoma negatiboen tratamendurako ere eraginkorra da. Ondorioz, gehien preskribatzen den antipsikotikoa da (Möller, 2005; Chopko eta Lindsley, 2018).

Paliperidona risperidonaren metabolito aktiboa da, eta profil farmakologiko bera duela frogatu da.



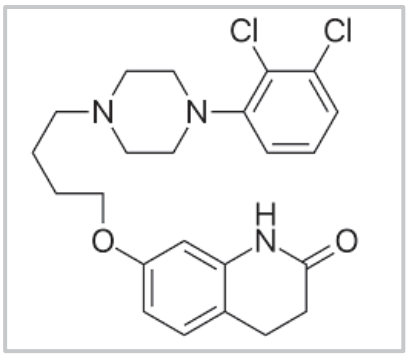

**1.14 Irudia:** Klozapina, olanzapina, quetiapina, risperidona eta paliperidonaren egitura kimikoa.

Azken urteotan, bigarren belaunaldiko antipsikotiko berriak garatu dira; hala nola asenapina eta lurasidona (Miyamoto et al., 2012). Hala ere, lehen belaunaldiko antipsikotikoen eraginkortasuna oraindik ez da hobetu. Gaur egun, eskizofrenia erresistentea tratatzeko onarturik dagoen botika bakarra klozapina da (Conley eta Kelly, 2001).

### 1.5.3 Hirugarren belaunaldiko antipsikotikoak

Honezkero, hirugarren belaunaldiko antipsikotikoak ere garatu dira; hala nola aripiprazola (**1.15 irudia**). Beste neuroleptiko batzuk ez bezala, hirugarren belaunaldiko antipsikotikoak ez dira $D_2$Ren antagonistak, $D_2$Ren agonista partzialak baizik (Davies et al., 2004). DA kontzentrazio handietan, DArekin lehiatzen dira, eragin klinikoa lortuz. Aitzitik, DAren mailak txikiak direnean, aripiprazola $D_2$R-rekin elkartu daiteke, eta agonista partzial gisa jardun.

Gainera, aripiprazola 5-$HT_1$Ren agonista partzial moduan aritzen da. Bigarren belaunaldiko antipsikotikoak ez bezala, aripiprazolak afinitate handiagoa du $D_2$Rekiko 5-$HT_{2A}$Rekin alderatuz (Chen et al., 2022). Hirugarren belaunaldiko antipsikotikoak sintoma psikotikoak arintzeko eraginkorrak dira, albo-ondorio estrapiramidalik eta hiperprolaktinemiarik eragin gabe. Gainera, pisu handitzea eta albo ondorio metaboliko gutxiago sortzen dituzte (Lieberman, 2004).

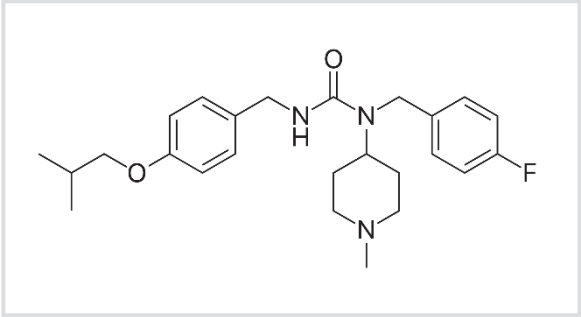



**1.15 irudia**: Aripriprazolaren egitura kimikoa

## 1.5.4 Antipsikotikoen belaunaldi berria

5-$HT_{2A}$Ren blokeoan oinarritutako eskizofrenia tratatzeko farmako berriak aurkitzeko ahaleginak, ritanserina eta blonanserina kasu, ez dira terapeutikoki erabilgarriak izan. 5-$HT_{2A}$Ren antagonista selektiboen erabilerak monoterapian ez zuenez eraginkortasun klinikoa aurkeztu, zenbait ikerketen arabera, 5-$HT_{2A}$Ren antagonismoa soilik ez da nahikoa antipsikotiko atipikoen eraginkortasun klinikoa azaltzeko (Miyamoto et al., 2012). Hala, badirudi $D_2$Ren blokeoa beharrezkoa dela eragin klinikoa lortzeko. Hala ere, pimabanserina deituriko 5-$HT_{2A}$Ren ligando potente eta selektiboa garatu zen psikosiaren tratamendurako alternatiba gisa (**1.16 irudia**) (Meltzer eta Roth, 2013). Pimabanserina afinitate dopaminergikorik ez duen onartutako lehen antipsikotikoa da (Hacksell et al., 2014). Orain arte, pimabanserina

eskizofreniarako terapian lagungarri gisa probatu da, haloperidola eta risperidona farmakoekin batera (Meltzer et al., 2012). Pimabanserinak FDAren onarpena lortu du parkinsonen gaixotasunean gertatzen diren eldarnioak eta haluzinazioak murrizteko, (Cummings et al., 2014). Gainera, pimabanserinak eskizofrenia duten pazienteen sintoma negatiboak murrizteko gaitasuna duela ere frogatu da (Bugarski-Kirola et al., 2022). Pimabanserina 5-$HT_{2A}$Ren alderantzizko agonista gisa deskribatu den arren, beharrezkoa da alderantzizko agonismoa frogatzeko esperimentu gehiago egitea (Vanover et al., 2006; Nutt et al., 2017).




**1.16 irudia:** Pimabanserinaren egitura kimikoa.

Berriki onartu da bigarren belaunaldiko antipsikotiko gehienak 5-$HT_{2A}$Ren alderantzizko agonistak direla, antagonista neutralak izan beharrean (Weiner et al., 2001). Antagonistek ez bezala, alderantzizko agonistek berezko eraginkortasun negatiboa dute, eta oinarrizko seinaleztapen-jarduera txikitu dezakete.

Oro har, antipsikotikoen ekintza-mekanismoa hobeto ulertzeak sendagai eraginkorrak eta jasangarriagoak diseinatu eta garatzea ekar lezake. Historikoki, profil polifarmakologikoa duten farmako promiskuoak eraginkorrak izan dira NSZren gaixotasunak tratatzeko, nahiz eta albo ondorio larri asko

eragin ditzaketen. Beraz, helburu molekular zehatzarekin elkarreragiten duten farmako selektiboen diseinuak sendagai eraginkorren eta onargarriagoen garapenean lagundu dezake. Hala balitz, agonismo alboratua edo selektiboa bakarka, zein alderantzizko agonismoarekin batera, aurkezten duten farmakoak, hartzaileen hautakortasunaren alternatiba gisa sortu ahalko lirateke, eskizofrenian espero diren erantzun funtzionalak lortzeko.

# Helburuak

Nahiz eta ikerketa asko egin diren sendagai antipsikotiko berriak garatzeko, gaur egun eskura dauden sendagaien artean ez da nabarmendu epe luzera eraginkortasuna duenik, albo-ondorio kaltegarririk gabe. Hori, eskizofreniaren neurobiologiari buruzko jakituria ezari zor zaio. Beraz, eskizofrenia maila neurobiologikoan sakonago ezagutuz gero, aldaketa molekularrak, zelularrak eta/edo seinaleztapen bideak identifikatu ahal izango dira, botika berrietarako itu terapeutiko berriak bilakatu daitezkenak.

Zenbait aurkikuntzek aditzera ematen dutenez, 5-$HT_{2A}$R sintoma psikotikoen eta horien tratamenduan eragiten duten mekanismo molekularretan inplikaturik dago. Alde batetik, psikodelikoen (LSD, psilozina, meskalina eta (±)DOI) izaera haluzinogenogenoa, 5-$HT_{2A}$Ren aktibazioan oinarritzen da. Bestetik, eskizofrenian erabili ohi diren antipsikotiko atipikoek 5-$HT_{2A}$Ren antagonista edo alderantzizko agonista gisa jokatzen dute.

Neuroirudi azterketek, *in vivo* PET eta *in vitro* post-mortem proben bidez, eskizofrenia duten subjektuetan 5-$HT_{2A}$R dentsitateari buruzko emaitza kontrajarriak erakutsi dituzte. Txosten kontrajarri horiek zerikusia daukate, antza, erabiltzen diren erradiotrazadore desberdinak hartzailearen konformazio desberdinetara finkatzearekin. Beraz, badirudi eskizofreniako 5-$HT_{2A}$Ren aldaketek zerikusi gehiago dutela hartzailearen egoera molekularraren aldaketekin hartzailearen adierazpen mailaren aldaketekin baino.

5-$HT_{2A}$R $G\alpha_{q/11}$- zein $G\alpha_{i/o}$-proteinak aktibatzeko gai da, hartzailera finkatzen den farmakoaren arabera. Testuinguru horretan, ezaugarri haluzinogenoen aztarna molekularra 5-$HT_{2A}$Ren agonista bidezko $G\alpha_{i1}$-proteinen aktibazioan oinarritzen da. Gainera, $G\alpha_{i1}$-proteinen estimulazio handiagoa deskribatu da, baina ez horrela $G\alpha_{q/11}$-proteinena; eskizofrenia duten subjektuen aurre-garunazalaren post-mortem ehunean eta 5-$HT_{2A}$Ren (±)DOI agonistaren presentzian egin zenean. Aurkikuntza hori, 5-$HT_{2A}$Ren agonismo alboratu gisa interpreta daiteke, eskizofrenikoetan $G\alpha_{i1}$-proteina bide haluzinogenoaren

bidez. Alde horretatik, $G\alpha_{i1}$-proteinek eragindako gainaktibitateak, eskizofrenian, 5-$HT_{2A}$Ren aktibitate handiagoa izatea eragingo luke. Giza garunean, 5-$HT_{2A}$Ren gainaktibitatea dagoela frogatzeko, 5-$HT_{2A}$Ren alderantzizko agonistak izango lirateke beharrezko tresnak.

Aurreko hipotesia berresten bada, 5-$HT_{2A}$Ren $G\alpha_{i1}$-proteinen alderantzizko agonista gisa jokatzen duten farmakoak tratamendu antipsikotikorako estrategia egokia izango lirateke, $G\alpha_{q/11}$-proteinengan duten jarduera intrintsekoa edozein dela ere. Bitxia bada ere, zenbait ikerketek iradokitzen dutenaren arabera, 5-$HT_{2A}$Rekiko afinitate handia duten antipsikotiko eraginkorrenetako batzuk 5-$HT_{2A}$Ren alderantzizko agonistak dira, antagonista neurtroak beharrean. Hala ere, antipsikotikoen ezaugarri farmakologikoei buruzko informazioa zeluletan egindako azterketen emaitzetan oinarritzen da, batez ere, eta jatorrizko ehunetan egindako azterketak, berriz, oso gutxi dira. Horrez gain, karakterizazio azterketa guztiak, oro har, medikamentu bakoitzaren selektibitate funtzionalaren profilari buruzko informaziorik gabeko seinaleztapen-bide bakarrera mugatzen dira.

Lan esparru horretan, ikerketaren lehen helburua aurretik antagonistatzat jotzen ziren 5-$HT_{2A}$Ren ligando diren hainbat sfarmakoren alderantzizko agonista ezaugarriak karakterizatzea izan zen. Alderantzizko agonistak erabiliz, 5-$HT_{2A}$Ren $G\alpha_{i1}$- eta $G\alpha_{q/11}$-proteinetara finkatzeko berezko aktibitate konstitutiboaren izaera frogatu zen.

Ondorengo helburua, 5-$HT_{2A}$Ren akoplamendu jarduera $G\alpha_{i1}$- eta $G\alpha_{q/11}$-proteinen bidez aztertzea izan zen, eskizofrenia zuten subjektuen giza post-mortem garunean.

Azkenik, bigarren belaunaldiko antipsikotikoen alderantzizko agonista ezaugarriak aztertu ziren, 5-$HT_{2A}$Ren akoplamendua aztertuz G-proteina desberdinetara.

# Subjektuak, Materialak eta Metodoak

## 3.1 Giza-garunaren laginak

### 3.1.1 Subjektuen aukeraketa, ezaugarri demografikoak, diagnosi psikiatrikoa eta analisi-toxikologikoa

Bilboko Auzitegi Medikuntzako Euskal institutuan giza garunak autopsia bidez lortu ziren. Laginen giza-jatorria ondokoen artean zegoen: beren buruaz beste egin zutenak, istripua jasandakoak, erailak edo heriotza naturala izan zutenak. Horiek lortzeko, post-mortem ikerketarako ezartzen diren hitzarmen politiko eta etikoak errespetatu ziren. Ikerlan osoa Euskal Herriko Unibertsitateko Gizakiekin lotutako Ikerketetarako Etika Batzordeak (GIEB) onartua izan zen (CEISH-UPV/EHU, Ref. M10-2019-230).

Forentseak heriotza baieztatu ostean, gorpuak gordeak izan ziren 4ºCtan autopsia egunerarte. Aurre-garunazal dortsolateral (DLPFC) laginak autopsia egunean bertan disekzionatu ziren, eta -80ºCtan gordeak izan ziren. Garun-eremuaren aukeraketarako, aurretik argitaratutako eskizofreniaren inguruko argitalpenen arabera izan zen (Lewis eta Gonzalez-Burgos, 2008; Sepherd et al., 2012; Kolk eta Rakic, 2022; Haber eta Robbins, 2022).

Odolaren azterketa akutuak (antidepresiboak, antipsikotikoak eta bestelako farmako psikotropikoak, opioideak eta etanola neurtzeko azterketa kuantitatiboak) Madrilgo Toxikologiako Institutu Nazionalean egin ziren, prozedura estandarrak jarraituz. Gainera, iraganean tratamendu antipsikotiko pean egondako pertsonen garun-laginetan ere egin ziren analisi toxikologikoak. Izan ere farmakoak, garunean odol plasman baino denbora luzeagoz metatzen dira , lipidotan eduki handiagoa izatearren. Horregatik, analisi horien helburua bereziki tratamenduan egondako pazienteen garun-laginetan antidepresiboak, antipsikotikoak, eta bestelako substantzia psikotropikoak (kotinina barne) bilatzea izan zen. Farmako antiepileptikoak, aldiz, ez ziren aztertuak izan analisi toxikologikoan (fenobarbital, fenitoina, lebetirazepam edo azido balproikoa). Analisi toxikologikoak Agilent

Technologies 1200 Series HPLC (Wilmington, DE, EEBB) gailuarekin egin ziren. Gailua 6410 Quad masen espektometro hirukoitzari lotu zitzaion, modu positiboan ioien iturri gisa ionizazio elektrospray (ESI) bat zuena, eta MassHunter softwarea erabiliaz.Horretarako, ZOBRAX (Agilent) Eclipse Plus C8 Narrow Bore (2.1 mm x 150 mm, 5 µm) zutabea aukeratu zen, eta zutabe-babesle gisa zutabearen eduki bera zuena erabili zen (Sampedro et al., 2012). Analisi toxikologiko guztiak Euskal Herriko Unibertsitateko (UPV-EHU) Ikerkuntzarako Zerbitzu Orokorretan (SGIker) burutu ziren.

Hiru talde esperimental aukeratu ziren immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika eta Western Blot azterketak egiteko: eskizofrenia taldea, eskizofrenikoak ez ziren suiziden taldea eta kontrol taldea. Subjektu guztiak ondoko parametroen arabera parekatu ziren azterketak egin aurretik, ahal zen heinean: adina, generoa eta post-mortem atzerapena (hil eta autopsia egin arteko denbora tartea; PMD) eta laginen metatze denbora. Diagnosirako eta heriotzaren kausa deskribatzeko irizpideak ondokoak dira:

**Eskizofrenia taldea**: Diagnosia irizpide internazionalak jarraituz (DSM IV, DSM IV-TR) egin zitzaien, eta beste edozein diagnosi psikiatriko baztertua izan zen. Taldea eskizofrenia pairatzen zuten 23 lagunek osatu zuten, eta ondoko ezaugarriak zituen: zazpi emakume eta hamasei gizon, batez besteko adina 48±4 urte, PMD 22±2 ordu, eta laginen metatze denbora 150±15 hilabete. Mediku aztertzaileak ondorioztatutakoaren arabera hamahiru eskizofreniko beren buruaz beste egiteagatik hil ziren; hamar jauziak eragindako traumaren ondorioz, bi urkatuak eta bestea uretan itota. Bere buruaz beste egiteagatik hil ez ziren hamar eskizofrenikoak ondokoagatik hil ziren; bihotzekoak jota (n=5), tumorea (n=1), odolustea (n=1), shock-ak jota (n=1), istripuz itota (n=1) eta altueratik erorita (n=1). Autopsia momentuan jasotako odolean ez zuten farmako antipsikotikoen arrastorik, analisi toxikologikoa burutu zitzaienean (**3.1 taula**).

**Eskizofrenikoak ez ziren suiziden taldea:** DMS irizpideen arabera diagnosi hauek izan zituzten: pertsonalitatearen asaldura (n=8), antsietate asaldura

(n=4) eta asaldura obsesibo-konpultsiboa (n=1). Taldea beren buruaz beste egin zuten 13 lagunek osatu zuten, eta ezaugarri hauek zituzten: emakume bakarra eta hamabi gizon, batez besteko adina 38±3 urte, PMD 16±2 ordu, eta laginen metatze denbora 207±15 hilabete. Heriotza-kausak hauek izan ziren: altuera batetik salto egitea (n=8), urkatuta itotzea (n=2), bere buruari tiro egitea (n=1), farmakoen gaindosia (n=1) eta uretan itotzea (n=1) (**3.1 taula**).

**Kontrol taldea:** Hil aurretik inongo gaixotasun neurologiko zein psikiatrikorik ez zuten subjektuen garun laginez osatutako tadea da. Hogeita hiru lagunek osatu zuten taldea, eta ezaugarri hauek zituzten: zazpi emakume eta hamasei gizon, batez besteko adina 48±3 urte, PMD 21±2 ordu, eta laginen metatze denbora 139±15 hilabete. Heriotza-kausak hauek izan ziren: trafiko-istripuak (n=12), porrot kardiobaskularra (n=4), altuera batetik erortzea (n=4), bihotzekoak jota (n=1), su-istripua (n=1) eta trenbideetara erortzeak eragindako trauma (n=1) (**3.1 taula**).

Garunen azterketa toxikologikoak baieztatu zuen kontrol taldean farmako psikotropiko eta antipsikotikoen presentziarik ez zegoela. Eskizofrenia ez zuten suiziden eta eskizofrenia taldeko kideek ez zutela farmako antipsikotikoen kontzentrazio aktiborik konfirmatu zen (**3.1 taula**).

**3.1 taula:** Eskizofrenia taldearen (**S**), eskizofrenia ez zuten suiziden taldearen (**NSS**) eta kontrol taldearen (**C**) ezaugarri demografikoak, *post-mortem* egoera, heriotza-kausa eta analisi toxikologikoaren emaitzak.

| Kasua | Diagnosia | Generoa (G/E) | Adina (urte) | PMI (ordu) | Metatzea (hilabete) | Heriotza-kausa | Heriotza-mekanismoa | Garun pH | Farmako maila odolean (mg/L) | Garun-toxikologia (ng/g) |
|---|---|---|---|---|---|---|---|---|---|---|
| **S 1** | Eskizofrenia | E | 67 | 22 | 17 | Naturala | Porrot kardiobaskularra | 5,8 | Negatiboa | Negatiboa |
| **C 1** | Kontrola | E | 66 | 17 | 83 | Istripua | Trafiko-istripua | 6,06 | Negatiboa | Ez egina |
| **S 2** | Eskizofrenia | G | 34 | 23 | 81 | Suizidioa | Alturatik salto | 6,32 | Negatiboa | Kotinina 23,1 |
| **C 2** | Kontrola | G | 34 | 17 | 69 | Istripua | Trafiko-istripua | 6,7 | Negatiboa | Negatiboa |
| **NSS 1** | Pertsonalitate asaldura | G | 34 | 7 | 199 | Suizidioa | Urkatua | Ez egina | Etanola 2700<br>Zitalopram 0,1<br>Oxakarbazepina 6,5 | Zitalopram 1579,9<br>Kotinina 3352,8<br>Norzitalopram 368,2 |
| **S 3** | Eskizofrenia | E | 53 | 18 | 98 | Naturala | Odulustea | 6,58 | Alprazolam 0,05 | Paliperidona 54,2<br>Kotinina 503,2<br>Alprazolam 43,9 |
| **C 3** | Kontrola | E | 51 | 10 | 70 | Naturala | Porrot kardiobaskularra | 6,3 | Negatiboa | Negatiboa |
| **S 4** | Eskizofrenia | G | 32 | 21 | 87 | Suizidioa | Altueratik salto | 6,65 | Negatiboa | Kotinina 380,78 |
| **C 4** | Kontrola | G | 33 | 23 | 95 | Istripua | Trafiko-istripua | 6,55 | Negatiboa | Ez egina |
| **NSS 2** | Pertsonalitate asaldura | G | 33 | 14 | 192 | Suizidioa | Altueratik salto | Ez egina | Lorazepam 0,03<br>Benlafaxina 0,16 | Desmetilbenlafaxine 475,0<br>Lorazepam 1421,2<br>Midazolam 3,7<br>Olanzapine 6 |
| **S 5** | Eskizofrenia | G | 45 | 36 | 15 | Istripua | Itota | Ez egina | Ez egina | Midazolam 1801,8<br>Nordiazepam 1247,1<br>Oxazepam 50,1 |
| **C 5** | Kontrola | G | 44 | 23 | 98 | Istripua | Trafiko-istripua | 6,45 | Negatiboa | Negatiboa |
| **NSS 3** | Pertsonalitate asaldura | G | 44 | 9 | 192 | Suizidioa | Altueratik salto | Ez egina | Etanola 220<br>Alprazolam 0,01<br>Amisulprida 1,4<br>Klomipramina 0,2<br>Reboxetina 0,09 | Alprazolam 141,4<br>Klomipramina 3040,7<br>Kotinina 322,5<br>Reboxetina 224,2 |
| **S 6** | Eskizofrenia | G | 49 | 23 | 107 | Istripua | Altueratik eroria | 6,40 | Negatiboa | Kotinina 391<br>Lorazepam 16,3 |
| **C 6** | Kontrola | G | 49 | 19 | 93 | Istripua | Trafiko-istripua | 6,7 | Negatiboa | Negatiboa |
| **S 7** | Eskizofrenia | G | 70 | 20 | 115 | Suizidioa | Urkatu | Ez egina | Ez egina | Negatiboa |
| **C 7** | Kontrola | G | 71 | 22 | 115 | Istripua | Altueratik eroria | 5,92 | Negatiboa | Negatiboa |
| **S 8** | Eskizofrenia | E | 74 | 9 | 118 | Naturala | Porrot kardiobaskularra | Ez egina | Fenobarbitala 9 | Negatiboa |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **C 8** | Kontrola | E | 74 | 30 | 167 | Istripua | Altueratik eroria | Ez egina | Negatiboa | Negatiboa |
| **S 9** | Eskizofrenia | G | 46 | 20 | 129 | Suizidioa | Altueratik salto | 6,41 | Negatiboa | Zuklopentixol 110,8<br>Kotinina 622,4<br>Lorazepam 25,5 |
| **C 9** | Kontrola | G | 46 | 22 | 115 | Naturala | Porrot kardiobaskularra | 6,48 | Negatiboa | Negatiboa |
| **NSS 4** | Pertsonalitate asaldura | G | 47 | 4 | 195 | Suizidioa | Altueratik salto | Ez egina | Fenitoina | Negatiboa |
| **S 10** | Eskizofrenia | G | 26 | 24 | 140 | Suizidioa | Altueratik salto | Ez egina | Diazepam 0,27 | Diazepam 356,5<br>Nor-diazepam 856,9<br>Oxazepam 15,1 |
| **C 10** | Kontrola | G | 25 | 21 | 71 | Istripua | Su-istripua | 6,48 | Negatiboa | Negatiboa |
| **NSS 5** | Pertsonalitate asaldura | G | 27 | 42 | 253 | Suizidioa | Altueratik saltot | Ez egina | Negatiboa | Kotinina 337,4<br>Diazepam 204,9<br>Nordiazepam 612<br>Oxazepam 65,8 |
| **S 11** | Eskizofrenia | E | 75 | 18 | 140 | Naturala | Porrot kardiobaskularra | Ez egina | Negatiboa | Kotinina 36,76 |
| **C 11** | Kontrola | E | 79 | 24 | 213 | Istripua | Trafiko-istripua | Ez egina | Negatiboa | Ez egina |
| **S 12** | Eskizofrenia | G | 28 | 28 | 143 | Suizidioa | Altueratik salto | Ez egina | Negatiboa | Kotinina 93,79 |
| **C12** | Kontrola | G | 29 | 13 | 116 | Istripua | Altueratik eroria | 6,44 | Negatiboa | Negatiboa |
| **NSS 6** | Pertsonalitate asaldura | G | 28 | 5 | 259 | Suizidioa | Altueratik salto | Ez egina | Negatiboa | Kotinina 638,2 |
| **S 13** | Eskizofrenia | G | 25 | 17 | 145 | Suizidioa | Altueratik salto | Ez egina | Negatiboa | Kotinina 153,1 |
| **C 13** | Kontrola | G | 23 | 16 | 141 | Istripua | Altueratik eroria | Ez egina | Negatiboa | Negatiboa |
| **NSS 7** | Alsaldura obsesibo-konpultsiboa | G | 26 | 19 | 143 | Suizidioa | Altueratik salto | 6,66 | Klomipramina 0,5<br>Fluoxetina 0,7<br>Fluboxamina 0,2<br>Ketiapina 0,5 | Fluoxetina 15441<br>Fluboxamina 4094<br>Norfluoxetina 4433,5<br>Norketiapina 386,7<br>Ketiapina 86 |
| **S 14** | Eskizofrenia | G | 23 | 13 | 195 | Suizidioa | Altueratik salto | Ez egina | Ez egina | Haloperidol 136,5<br>Kotinina 458,5<br>Ketiapina 392,5<br>Norketiapina 1309,4 |
| **C 14** | Kontrola | G | 22 | 20 | 188 | Istripua | Trafiko-istripua | Ez egina | Nordiazepam 0,38 | Kotinina 371,6<br>Oxazepam 80,9<br>Nordiazepam 909 |
| **NSS 8** | Pertsonalitate asaldura | G | 19 | 8 | 131 | Suizidioa | Altueratik salto | 6,7 | Negatiboa | Kotinina 236,4 |
| **S 15** | Eskizofrenia | E | 80 | 32 | 178 | Naturala | Shock | Ez egina | Negatiboa | Ez egina |
| **C 15** | Kontrola | E | 78 | 12 | 185 | Naturala | Porrot kardiobaskularra | Ez egina | Negatiboa | Ez egina |
| **S 16** | Eskizofrenia | E | 38 | 23 | 216 | Suizidioa | Altueratik salto | Ez egina | Negatiboa | Negatiboa |
| **C 16** | Kontrola | E | 36 | 19 | 110 | Istripua | Trenbidera eroria | 6,51 | Negatiboa | Negatiboa |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **NSS 9** | Antsietate asaldura | E | 39 | 19 | 214 | Suizidioa | Farmako gaindosia | Ez egina | Nordiazepam 0,13<br>Etanola 5000 | Diazepam 22,4<br>Nordiazepam 280,7 |
| **S 17** | Eskizofrenia | E | 51 | 15 | 236 | Naturala | Porrot kardiobaskularra | Ez egina | Buflomedil 66<br>Metamizol 4 | Kotinina 521,5 |
| **C 17** | Kontrola | E | 51 | 38 | 230 | Istripua | Trafiko-istripua | Ez egina | Negatiboa | Ez egina |
| **S 18** | Eskizofrenia | G | 62 | 28 | 240 | Suizidioa | Altueratik salto | Ez egina | Ez egina | Amitriptilina 170,3<br>Nortriptilina 453 |
| **C 18** | Kontrola | G | 62 | 23 | 243 | Istripua | Trafiko-istripua | Ez egina | Negatiboa | Ez egina |
| **NSS 10** | Moldaera antsietate asaldura | G | 62 | 19 | 271 | Suizidioa | Crushing | Ez egina | Ez egina | Maprotilina 257,9<br>Nordiazepam 1584,9 |
| **S 19** | Eskizofrenia | G | 35 | 22 | 299 | Suizidioa | Altueratik salto | Ez egina | Negatiboa | Negatiboa |
| **C 19** | Kontrola | G | 36 | 22 | 286 | Istripua | Trafiko-istripua | Ez egina | Etanol 1000 | Ez egina |
| **NSS 11** | Antsietate asaldura | G | 36 | 15 | 181 | Suizidioa | Arma tiroa | Ez egina | Mirtazapina 0,08 | Ez egina |
| **S 20** | Eskizofrenia | G | 49 | 41 | 292 | Suizidioa | Urkatua | Ez egina | Etanola 490<br>Ez egina | Kotinina 127<br>Klorpromazina 140,7<br>Lorazepam 388,6<br>Tioridazina 6079,5 |
| **C 20** | Kontrola | G | 45 | 30 | 275 | Istripua | Trafiko-istripua | 6,82 | Etanola 3090 | Ez egina |
| **NSS 12** | Antsietate asaldura | G | 46 | 26 | 319 | Suizidioa | Urkatua | Ez egina | Ez egina | Diazepam 91,3<br>Nordiazepam 175,2 |
| **S 21** | Eskizofrenia | E | 56 | 24 | 125 | Naturala | Porrot kardiobaskularra | Ez egina | Alprazolam 0,03 | Kotinina 110<br>Alprazolam 38 |
| **C 21** | Kontrola | E | 54 | 24 | 10 | Istripua | Altueratik eroria | 6,87 | Negatiboa | Negatiboa |
| **S 22** | Eskizofrenia | G | 50 | 3 | 173 | Suizidioa | Uretan itota | 7,09 | Nordiazepam 0,4 | Amisulprida 75,9<br>Kotinina 89<br>Nordiazepam 662,8<br>Oxazepam 47,6<br>Trazodona 241,6 |
| **C22** | Kontrola | G | 50 | 2 | 15 | Naturala | Porrot kardiobaskularra | 6,1 | Negatiboa | Negatiboa |
| **NSS 13** | Pertsonalitate asaldura | G | 49 | 22 | 141 | Suizidioa | Altueratik eroria | 6,35 | Nordiazepam 2,8<br>Tiaprida 5,4<br>Benlafaxina 1,2 | Kotinina 254,1<br>Desmetillbenlafaxina 265,6<br>Oxazepam 94,7<br>Tiaprida 973,5<br>Benlafaxina 2146,5 |
| **S 23** | Eskizofrenia | G | 43 | 17 | 172 | Naturala | Porrot kardioaskularra | Ez egina | Negatiboa | Kotinina 1003,9<br>Lorazepam 66 |
| **C 23** | Kontrola | G | 41 | 15 | 7 | Naturala | Porrot kardiobaskularra | Ez egina | Negatiboa | Negatiboa |

Ezaugarri demografikoak, adina, PMD, metatze denbora eta pH-a, 3.2 taulan laburtuak daude. Konparaketa estatistikoak egin zirenean ez zen diferentziarik aurkitu, aztertutako hiru taldeen artean, adinak alderatzean (F[2,56]=2,08, p=0.1341). Era berean, ez zen diferentzia esanguratsurik aurkitu hiru taldeen artean PMD aztertu zenean (F[2,56)]=1,99, p=0,1456), ezta pH-a aztertu zenean ere (F[2,21]=0,17, p=0,8433). Hala ere, eskizofrenia taldearen laginen metatze denbora luzeagoa izan zen kontrol taldearekin eta eskizofrenia ez zuten suiziden taldearekin konparatuz (F[2,56]=4,66, p=0,0135) (3.2 taula).

**3.2 taula:** Ikerlanean aztertutako eskizofrenia taldearen, eskizofrenia ez zuten suiziden taldearen ezaugarri demografikoak, nahiz post-mortem ezaugarriak. Talde bakoitzaren batezbestekoa±SEM (batezbestekoaren errore estandarra):

| Taldea | Generoa | Adina(urteak) | PMD | pH | Metatze denbora (hilak) |
|---|---|---|---|---|---|
| Eskizofrenia | 7 E/16 G | 48±4 | 22±2 | 6,5±0,1 | 150±15 |
| Eskizofrenia ez zuten suizidak | 1 E/12 G | 38±3 | 16±3 | 6,6±0,1 | 207±15* |
| Kontrol | 7 E/16 G | 48±3 | 21±2 | 6,5±0,1 | 139±15 |

*p<0,05 vs kontrol taldea (Bonferroniren konparazio anizkoitzaren azterketa)

### 3.1.2 Immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika (SPA) eta Western Blot frogen karakterizaziorako erabili zen lagin multzoa osatzen zuten partaideen ezaugarri demografikoak.

Farmakoen hasierako karakterizazio farmakologikoa DLPFC lagin multzo batekin egin zen, gainontzeko frogetan erabili ez zirenetatik abiatuta. Lagin multzoen mintzak prestatzeko sei subjektuen aurre-garun-azal zatiak homogeneizatu ziren. Guztira, 2016 eta 2019 bitartean jasotako hamabost lagin erabili ziren ikerlan osorako (%30 gizonezkoak eta %70 emakumezkoak), non batezbesteko adina 59±6 urtekoa zen, PMD 11±2 ordukoa eta metatze denbora 27±3 hilekoa zen. Partehartzaileen odolean egindako proba toxikologikoetan lortutako emaitzen arabera, ez zeukaten farmako psikotropikorik hil zirenean.

## 3.2 Animaliak: Sagu transgenikoak

Lan honetan erabilitako 5-$HT_{2A}$R *knock-out* saguak (5-$HT_{2A}R^{(-/-)}$) eta jatorrizko edo *Wild Type* saguak (5-$HT_{2A}R^{(+/+)}$) R. Maldonado katedradunak (Bartzelona, Espainia) eskuzabaltasunez emanak izan ziren. Sagu transgenikoak lehenengo 129S6/SvEv anduian sortu ziren, eta ondoren C57BL/6J anduiarekin gurutzatu ziren, laborategiko prozedura estandarrak jarraituz (González-Maeso et al., 2003; Weisstaub et al., 2006; González-Maeso et al., 2007; Orejarena et al., 2011). Animaliak jaso ostean, gurutzatze gehigarriak egin ziren kolonia freskatzeko. Gure laborategian ohiko polimerizazio-katearen erreakzioak (PCR) eta elektroforesiak egin ziren gure saguak genotipatzeko (ez dira emaitzak sartu), aurretik argitaratutako prozedurak jarraituz (Fiorica-Hollowells et al., 2002). 5-$HT_{2A}R^{(-/-)}$ saguek 5-$HT_{2A}$R espresatzen ez zutela egiaztatzeko, [$^3$H]ketanserinarekin finkapen azterketak (Muguruza et al., 2013) eta (±)DOIk eragindako $G\alpha_{i1}$/$G\alpha_{q/11}$-proteinen aktibazio azterketak (Garcia-Bea et al., 2019) egin ziren.

Horretarako, C57BL/J6 sagu heldu arrak erabili ziren (15-20 asteko adinarekin). Animaliok bosnaka taldekatu ziren kaioletan, ohikoak diren laborategiko baldintzapean (22±1°C, %55±5 hezetasun erlatiboa, 12 h argi/ilun zikloa eta askatasun osoa ohiko marraskarien janaria eta ura lortzeko). Erabilitako animalien zenbatekoa murrizteko ahalegina egin zen. Gainera, jarraitutako esperimentuen protokoloak Euskal herriko Unibertsitateko (UPV-EHU) Animaliekin egiten den Esperimentaziorako Etika Batzordetik (AEEB) onartuak izan dira (Erreferentzia: M20-2019-321). Era berean, esperimentu guztietan jarraitutako prozedurek Europar Batasuneko (European Union Directive 2010/63/UE) eta Espainiako (53/2013 errege dekretua) legediek ezartzen dituzten arauak betetzen dituzte, animalien ongizateari dagokionean. Sagu helduak lepo dislokazio bidez hil ziren, ondoren garuna atera zitzaien eta garun-azala disekzionatu (Diez-Alarcia et al., 2016). Laginak -80°Ctan gorde ziren erabiliak izan arte.

## 3.3 Farmakoak

-(±)-DOI: (±)-2,5-Dimetoxi-4-iodoamfetamina hidrokloruroa (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Ketanserina: 3-[2-[4-(4-Fluorobentzoil)-1-piperidinil]etil]-2,4[1*H*,3*H*]-quinazolinadiona tartratoa (Tocris; Bristol, Erresuma Batua).

-MDL100907 (bolinanserina): (R)-(+)-α-(2,3-dimetoxifenil)-1-[2-(4-fluorofenil)etil]-4-pipidin metanola (Sigma-Aldrich; Saint Louis, Missouri, USA).

-Altanserina: 3-[2-[4-(4-Fluorobentzoil)-1-piperidinil]etil]-2,3-dihidro-2-tioxo-4(1H)-kinazolinona hidrokloruro hidratoa (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Pimabanserina (ACP-103): 1-(4-Fluorobentzil)-3-(4-isobutoxibentzil)-1-(1-metilpiperidin-4-il)urea (Axon Medchem; Groningen, Herbehereak).

-Nelotanserina: 1-(3-(4-bromo-2-metil-2H-pirazol-3-il)-4-metoxifenil)-3-(2,4-difluorofenil)urea (Axon Medchem; Groningen, Herbehereak).

-Ritanserina: 6-[2-[4-[bis(4-fluorofenil)metilideno]piperidin-1-il]etil]-7-metil-[1,3]tiazolo[3,2-a]pirimidin-5-ona (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Eplibanserina: 4-[(*E*,3*Z*)-3-[2-(dimetilamino)etoximino]-3-(2-fluorofenil)prop-1-enil]fenola (Axon Medchem; Groningen, Herbehereak).

-MDL-11,939: α-fenil-1-(2-feniletil)-4-piperidin metanola (Tocris; Bristol, Erresuma Batua).

-SB 242084: 6-Kloro-2,3-dihidro-5-metil-*N*-[6-[(2-metil-3-piridinil)oxi]-3-piridinil]-1*H*-indol-1-carboxiamida dihidrokloruroa (Tocris; Bristol, Erresuma Batua).

-Klozapina: 8-Kloro-11-(4-metil-1-piperazinil)-5H-dibentzo[b,e][1,4]diazepina (Tocris; Bristol, Erresuma Batua).

-Risperidona: 3-[2-[4-(6-fluoro-1,2- bentzisoxazol-3-il)-1-piperidinil]etil]-6,7,8,9-tetrahidro-2-metil-4H-pirido[1,2- a]pirimidin-4-ona (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Aripiprazola: 7-{4-[4-(2,3-Diklorofenil)-1-piperazinil]butoxi}-3,4-dihidro-2(1H)-quinolinona (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Olanzapina: 2-Metil-4-(4-metil-1-piperazinil)-10H-tieno[2,3-b][1,5]bentzodiazepina (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Paliperidona: 3-[2-[4-(6-Fluoro-1,2-bentzisoxazol-3-il)-1-piperidinil]etil]-6,7,8,9-tetrahidro-9-hidroxi-2-metil-4*H*-pirido[1,2-*a*]pirimidin-4-ona (Tocris; Bristol, Erresuma Batua).

-Ketiapina: 2-[2-(4-Dibentzo[*b*,*f*][1,4]tiazepin-11-il-1-piperazinil)etoxi]etanol hemifumaratoa (Tocris; Bristol, Erresuma Batua).

-Atropina: Azido endo-(±)-α-(hidroximetil)bentzeno azetikoaren 8-metil-8-azabiziklo[3.2.1]okt-3-il esterra (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Fentolamina: 2-[N-(3-Hidroxifenil)-p-toluidinometil]-2-imidazolidina hidrokloruroa (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Zetirizina: Azido [2-[4-[(4-klorofenyl)fenilmetil]-1-piperazinil]etoxi]azetikoaren hidrokloruroa (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

-Raclopridea: 3,5-Dikloro-N-[[(2S)-1-etil-2-pirrolidinil]metil]-2-hidroxi-6-metoxibentzamida (Tocris; Bristol, Erresuma Batua).

-Haloperidola: 4-[4-(4-klorofenil)-4-hidroxipiperidino]-4′-fluorobutirofenona (Sigma-Aldrich; Saint Louis, Missouri, EEBB).

## 3.4 Materialak

**Antigorputzak:**

Immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika (SPA) eta Western Blot frogak egiteko erabili ziren antigorputz monoklonalak Santa Cruz Biotechnology, Inc (Kalifornia EEBB) enpresari erosi zitzaizkion. Antigorputzen ezaugarriak **3.5.2 atalean** zehaztuak daude, non Western Blot esperimentuen protokoloa ere azaltzen den.

Western Blot frogetan bi antigorputz sekundario fluoreszente ezberdin erabili ziren: bata gorria, anti-sagu antigorputzarekin elkartutako Alexa Fluor® 680 (Invitrogen, Oregon, EEBB), eta bestea berdea, anti-untxi antigorputzarekin elkartutako IRDye™ 800 (Rockland Immunochemical, Pennsylvania, EEBB).

**Konposatu erradiaktiboak:**

Azufre 35az markatutako guanosina-5'-*O*-(gamma-tio)-trifosfatoa ([$^{35}$S]GTPγS), aktibitate espezifikoa 1250 Ci/mmol zuena eta PerkinElmer laborategiari erosia (Waltman, MA, EEBB).

**Bestelako farmako eta konposatu kimikoak:**

-Bio-Rad Laboratories (California, EEBB): Amonio persulfatoa (APS), Bradford Protein Assay, Laemmli laginen tanpoia 2 aldiz kontzentratua, N-N-N-N´-tetrametiletilenediamina (TEMED), aurrez markatutako SDS-PAGE pisu molekularraren estandarrak.

-Carlo Erba Erreaktiboak (Bartzelona, Espainia): Metanola.

-GE Healthcare (Buckinghamsire, Erresuma Batua): Nitrozelulosa mintzak (poro tamaina: 0.45 µm) eta Whatman™ zelulosa 3MM.

-Invitrogen (Bartzelona, Espainia): DL-Ditiotreitola (DTT), azido etilenodiamino tetrazetikoa (EDTA).

-National diagnostics (Atlanta, GA, EEBB): %30 akrilamida 30-%0,8 bisacrilamida.

-Panreac S.A.U (Bartzelona, Espainia): Azido azetiko glaziala, azukrea eta HCl (%37).

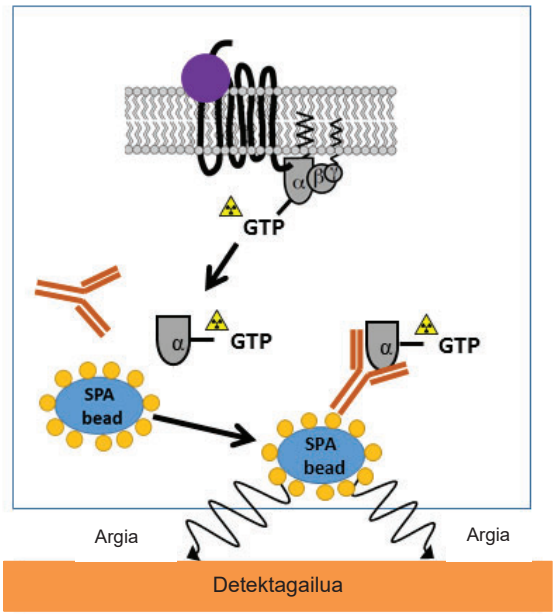
-Sigma-Aldrich® (Saint Louis, Missouri, EEBB): Behiaren serumeko albumina (BSA), 2-butanola, dimetilsulfoxidoa (DMSO), azido etileno glikol-bis(2-aminoetilether)-N,N,N′,N′-tetraazetikoa (EGTA), glizina, guanosina difosfatoa (GDP), guanosina 5'-O-[gamma- tio]trifosfatoa (GTPγS), Igepal® CO-520, β-merkaptoetanola, $MgCl_2$, NaCl, NaF, $Na_3VO_4$, Proteasa Inhibitzaile Cocktail, polioxietilenoa (20) sorbitan monolauratoa (Tween™ 20), sodio deoxikolatoa (SDC), sodio dodezil sulfatoa (SDS), Tris (hidroximetil)aminometano hidrokloruroa (Tris HCl).

-Perkin Elmer (Waltham, MA, EEBB): 96 putzudun isoplaka eta A proteina itsatsirik duten polibiniltoluenozko (PVT) SPA bolatxoak.

## 3.5 Metodoak

### 3.5.1 Immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika (SPA)

Hartzaileek modu espezifikoan aktibatzen dituzten Gα-proteinak zehazteko, immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika erabili zen. Horrela, antigorputzak erabiltzen dira Gα-proteina espezifikoak ezagutzeko, eta [$^{35}$S]GTPγS harrapatzean sortutako energia izarniadura gisa detektatzen da. Teknika horren bidez, neurtu ahal da farmako batek eragindako erantzuna Gα-proteina espezifikoen bidez. Horretarako, Gα-proteinek [$^{35}$S]GTPγS harrapatzeko duten ahalmenaz baliatzen gara, GTPren analogoa baita, baina [$^{35}$S]GTPγS hidrolizaezina den molekula izanik, itsatsia geratzen zaio Gα-proteinari. [$^{35}$S]GTPγS SPA, beraz, azterketa funtzionala da, non farmako baten eraginkortasuna eta potentzia detektatu eta kuantifikatzeko baliagarria den, sistema jakin batean. Horren bidez, agonista edo alderantzizko agonisten eraginkortasun maximoa kalkulatu daiteke ($E_{max}$ edo $I_{max}$, hurrenez hurren), asintotaren balio maximoari dagokiona. Era berean, eragin maximoaren %50 eragiteko gai den farmakoaren kontzentrazioa izango da potentziaren adierazle, estimulazioa edo inhibizioa izanik ($EC_{50}$/$IC_{50}$).




**3.1 irudia:** Immunoprezipitazioarekin akplatuako [$^{35}$S]GTPγS finkapen teknikaren adierazpen grafikoa 96 putzutako xafla bateko edozein putzutan aurki daitekeen nahastea, agonista bidez gertatzen den edozein GPCRren aktibazioa adierazten duena. Gα subunitea aktibatzen denean, mintzetik banatzen da detergenteen presentzian, [$^{35}$S]GTPγS lotzen zaio, eta anti-Gα antigorputzak ezagutu eta lotuko du. Energia erradiaktiboaren zehaztea gerta dadin, erradioaktiboa den molekula polimeroz osatutako bolatxo disdiratsuei gerturatu behar zaie, hauek izarniadura eman dezaten. Izarniadura bolatxoek G-proteina-antigorputz konplexua harrapatzen dutenean, erradiaktibitatea fotoi bilakatzen da izarniadurari esker, eta hori neurgarria da izarniadura kontagailuei esker. C. Muruguza tesirako sortutako ilustrazioa, *DeLapp NW. 2004tik* moldatua.

### 3.5.1.1 Mintzetan aberastutako laginen prestaketa ($P_2$ zatikia)

Mintzen laginen prestakuntzarako, aurretik deskribatua zegoen prozedura jarraitu zen, egindako moldaketak minimoak izanik (González-Maeso et al., 2000). Garun laginak (giza garunaren 1g edo saguaren 200mg) emeki birrinduak eta homogenizatu ziren, 4ºCtan, Teflon-beirazko birringailua erabiliz (10 gora-behera joaldi 15000 rpmtara) eta 30 homogenizazio tanpoi bolumenetan (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$ eta 1 mM DTT, pH 7,4; 250 mM azukre gehituta). Lagin homogenizatuak 5 minutuz zentrifugatu

ziren (Sorvall RC-5C zentrifuga, SM-24 errotorea; FisherScientific, Madril, Espainia) 1000 x g aplikatuz (4°C). Lortutako pelletak baztertu ziren eta gainjalkina berriro zentrifugatu zen 10 minutuz 40000 x g aplikatuz (4°C). Hemendik lortutako pelleta 20 bolumen zentrifugazio tanpoitan bereseki ziren (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$ eta 1 mM DTT; pH 7.4), eta berriro zentrifugatu aurreko baldintzetan. Ondoren, pelletak 5 bolumen zentrifugazio tanpoitan bereseki ziren. Laginen proteina edukia zehaztu ostean, 0,5 mg zeukaten laginak prestatu eta 21000 x g-tan zentrifugatu ziren (Eppendorf 5810R zentrifuga, Eppendorf, Madril, Espainia) 15 minutuz (4°C). Lortutako gainjalkina baztertu zen eta pelletak erabili ziren arte 80ºCtan gorde ziren.

Laginen proteina edukia zehazteko Bradford Metodoa (1976) jarraitu zen, behien serumeko albumina (BSA) estandar gisa erabilita (González-Maeso et al., 2000). Horretarako, 200 µl Bradford Proteina frogarako erreaktiboaren 1:5 diluzioa erabili zen, MilliQ urarekin prestatua, eta 10 µl proteina lagin gehitu zitzaizkion (1:3 eta 1:4 diluzioak). Giro tenperaturan 6 minutuz inkubatu ostean, disoluzioaren absorbantzia neurtu zen 630 nm uhin luzeran ELX808 mikroxafla irakurgailua erabiliz. BSA estandard gisa erabili zen, 0 eta 0,5 mg/ml tartean, laginen proteina edukia estrapolatzeko.

### 3.5.1.2 Immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika (SPA)

5-$HT_{2A}$Rek espezifikoki aktibatu ahal dituzten Gα-proteinak zehazteko protokolo berezia erabili zen; immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknika (SPA), hain zuzen. Horren oinarria [$^{35}$S]GTPγS finkapen froga eta antigorputz espezifikoak nahastean datza. Horretarako, aurretik argitaratua zegoen prozedura erabili zen, egindako moldaketak minimoak izanik (Erdozain et al., 2012; Diez-Alarcial et al., 2016; Moreno et al., 2016; Ibarra-Lecue et al., 2018; Garcia-Bea et al., 2019; Muneta-Arrate et al., 2021; Brocos-Mosquera et al., 2021; Diez-Alarcia et al., 2021b).

Lehendabizi, mintzetan aberastutako laginak emeki birrindu eta SPA inkubazio tanpoian bereseki ziren, 4ºCtan (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT eta 100 mM NaCl; pH 7,4), lortutako laginen proteina edukia 68,75 µg/ml izan arte. [$^{35}$S]GTPγS finkapen frogak 96 putzutako xafletan egin ziren, non putzu bakoitzean (200 µl putzuko) ondokoa nahastu zen: SPA disoluzioa (SPA inkubazio tanpoia/DMSO), 0,4 nM [$^{35}$S]GTPγS (1250 Ci/mmol), 11 µg proteina, eta hainbat GDP kontzentrazio (100-50 µM), Gα-proteinen subunitatearen arabera aukeratua (**3.3 taula**). Espezifikoa ez den finkapena zehazteko (NSB), markatu gabeko GTPγS gehitu zitzaion, kontzentrazio handian (100 µM) (**3.4 taula**).

**3.3 taula:** Immunoprezipitazioarekin akoplatutako [$^{35}$S]GTPγS finkapen teknikan (SPA) erabilitako GDP kontzentrazioak eta antigorputzen diluzioak.

| Itual | [GDP] | Antigorputz diluzioa |
|---|---|---|
| $G\alpha_{i1}$ | 100 µM | 1:20 |
| $G\alpha_{i2}$ | 50 µM | 1:20 |
| $G\alpha_{i3}$ | 100 µM | 1:60 |
| $G\alpha_{o}$ | 50 µM | 1:40 |
| $G\alpha_{q/11}$ | 50 µM | 1:40 |

Garun mintzak 30 minutuz eta 30ºCtan farmakoekin aurreinkubatu ziren, farmako guztiak markatze-epean orekan daudela ziurtatzeko asmoz. Ondoren, 90 minutuz inkubatu ziren mintzak (30ºC), emeki irabiatuz (400 rpm). Horretarako, 20 µl-ko detergente disoluzio gehitu zitzaien (1% Igepal, 0,1% sodio deoxikolatoa, 1% proteasa inhibitzaile koktail) mintzak disolbatzeko, baina [$^{35}$S]GTPγS-Gα-proteina elkartea hautsi gabe. Aldi berean, proteasen inhibitzaileei esker, proteasek denboran zehar seinalea gutxitzea ekidin zen (Harder eta Fotiadis, 2013). Mintzak disolbatu ostean, Gα subunitate bakoitzarentzat espezifikoa den 10 µl antigorputz diluzio gehitu zen putzu bakoitzean **(3.3 taula)**. Antigorputzak aurrez disolbatuak izan ziren %15 BSA

kontzentrazio ezberdinetan eta Gα-proteina azpimota bakoitzarentzat (Diez-Alarcia et al., 2021). Azkenik, 90 minututako inkubazioaren ondoren, 50 µl PVT A proteina SPA bolatxoak gehitu ziren, 0,75 mg/ml kontzentrazioa zutenak, eta xafla 180 minutuz inkubatu zen (22°C), emeki irabiatuz (350 rpm) (**3.4 taula**). Nahastea inkubatzean A proteina duten bolatxoak eta aurretik Gα-proteina lotua zuten antigorputzen elkarketa bultzatzea lortzen da (Ferrer *et al*., 2003). Xafla bakoitza *MicroBeta TriLux scintillation counter* detektagailuan neurtu aurretik (PerkinElmer España, S.L., Madril, Spain) zentrifugatu egin zen (15 minutuz 1000 x g-tan).

**3.4 taula:** Immunoprezipitazioarekin akoplaturiko [$^{35}$S]GTPγS finkapen teknikaren protokolo orokorra. **BB**: Finkapen basala (farmakorik gabe neurtzen den finkapenaren balioa), **NBS**: finkapen ez espezifikoa (neurtzen den [$^{35}$S]GTPγS finkapena makatu gabeko 100 μM GTPγS gehitzean).

| | **BB** | **Agonista** | **Agonista + Antagonista** | **NBS** |
|---|---|---|---|---|
| **SPA inkubazio tanpoia/DMSO** | 24 μl | 12 μl | / | 12 μl |
| **GTPγS** | / | / | / | 12 μL |
| **GDP** | 12 μl | 12 μl | 12 μl | 12 μl |
| **Antagonista** | / | / | 12 μl | / |
| **Mintzak** | 160 μl | 160 μl | 160 μl | 160 μl |
| 15 minutuz inkubatu 30°Ctan, emeki irabiatuz (400 rpm) | | | | |
| **Agonista** | / | 12 μl | 12 μl | / |
| 30 minutuz inkubatu 30°Ctan, emeki irabiatuz (400 rpm) | | | | |
| **[$^{35}$S]GTPγS** | 5 μl | 5 μl | 5 μl | 5 μl |
| 90 minutuz inkubatu 30°Ctan, emeki irabiatuz (400 rpm) | | | | |
| **Detergentea** | 20 μl | 20 μl | 20 μl | 20 μl |
| 30 minutuz inkubatu 22°Ctan, emeki irabiatuz (350 rpm) | | | | |
| **Antigorputzak** | 10 μl | 10 μl | 10 μl | 10 μl |
| 90 minutuz inkubatu 22°Ctan, emeki irabiatuz (350 rpm) | | | | |
| **PVT A proteina SPA Bolatxoak** | 50 μl | 50 μl | 50 μl | 50 μl |
| 180 minutuz inkubatu 22°Ctan, emeki irabiatuz (350 rpm) | | | | |

Oro har, 5-HT$_{2A}$Ren alderantzizko agonista selektiboak aurkitzeko helburuarekin, aurretik antagonista gisa deskribatuak zeuden farmakoak aukeratu ziren, eta horien alderantzizko agonista gisako ezaugarriak aztertu ziren. Ondoren, alderantzizko agonista gisa jokatzen zuten farmakoak erabili ziren eskizofrenia zuten gaixoetan 5-HT$_{2A}$Ren eta Gα-proteinen arteko akoplamendua aztertzeko. Gainera, erabilitako farmakoen ezaugarriak espezifiko zein selektiboak zirela ziurtatzeko, 5-HT$_{2A}$R *knock-out* sagu transgenikoak eta antagonista neutroak erabili ziren. Horietaz baliatuz,

farmako bakoitzak [$^{35}$S]GTPγS finkapenean neurtutako seinalea blokeatzea lortu zen.

### 3.5.1.3 Emaitzen analisi matematikoa eta estatistikoa

#### Analisi matematiko

Microbeta Trilux Scintillation counter detektagailuan lortutako emaitzak CCPM (minutuko zuzendutako kontu) gisa espresatuak daude, non CCPMak eta DPMak (minutuko desintegrazioak) baliokideak direla onartzen den. Emaitzak interpretatzeko, CCPMtan dauden datuak finkatutako [$^{35}$S]GTPγS fentomol proteina miligramoko unitatetan bilakatu behar dira (fmol/mg proteina). Datuen bilakatzea egiteko, azterketan erabilitako proteina kontzentrazioa kontuan hartu behar da, eta **3.1 ekuazioa** betetzen da**.** [$^{35}$S]GTPγS finkapenean proteina kontzentrazioak eragina izan dezake Diez-Alarcia *et al.,* 2021 artikuluan frogatu zen moduan. Horregatik froga horietan proteina edukia neurtzen da esperimentu bakoitzeko.

**fmol/ mg proteina**: CCPM kontuak/(2,22 x 1250 x [frogako proteina kontzentrazioa mg])

**3.1 ekuazioa**: [$^{35}$S]GTPγS finkapena eta SPA dituzten esperimentuetan lortutako emaitzak fmol/mg proteinako. 1250 (Ci/mmol) $^{35}$S erradioligandoak berezkoa duen aktibitate espezifikoari dagokio, 2,22 balioa konstantea da Curie (Ci) unitateak DPMtan bilakatzeko. (1 Ci=2,22x10$^{12}$ DPM).

[$^{35}$S]GTPγSren finkapen basala (BB), farmako exogenorik ezean gertatzen den [$^{35}$S]GTPγSren finkapenari dagokio. Agonista edo alderantzizko agonista gehitzean [$^{35}$S]GTPγSaren finkapen basala modulatua izango da. Izan ere, agonista gehitzean [$^{35}$S]GTPγSaren finkapena handitzen da, jatorrizko [$^{35}$S]GTPγS finkapen basalarekiko. Aldiz, alderantzizko agonista gehitzean [$^{35}$S]GTPγSaren finkapen basala txikitzen da.

Bestalde, finkapen ez espezifikoa (NBS) CCPMtan lortzeko, egoera esperimental bakoitzetik kenketa eginez atera zen; finkapen basala, estimulazioa, inhibizioa, etab. Hortaz, balio bakoitzari finkapen ez espezifikoa kentzean, [$^{35}$S]GTPγSaren finkapen espezifikoa lortu zen; kalkuluak eta analisi estatistikoa egiteko erabiliko zena.

Farmako agonista, antagonista edo alderantzizko agonisten ezaugarriak ezagutzeko, kontzentrazio-erantzun kurba bat lortu zen farmako bakoitzarentzat. Horretarako, farmako gabe lortutako finkapen basalaren balioa finkapenaren %100 gisa onartu zen. Ondoren, farmakoarentzat lortutako finkapen espezifikoa portzentai gisa bilakatu zen. Horrela, lortutako [$^{35}$S]GTPγS finkapenaren estimulazioa edo inhibizioa finkapen basalaren arabera adierazi zen. Kurba osatzen duten puntu bakoitzaren kalkulurako **3.2** eta **3.3 ekuazioak** erabili ziren:

%Estimulazioaren: (Estimulazioa-NBS)/(BB-NBS)x100

**3.2 ekuazioa**: [$^{35}$S]GTPγS finkapena SPArekin konbinatzean lortutako emaitzen kalkulua finkapen basalarekiko portzentai izaera agonista, antagonista edo lehiakorra deskribatzeko. **NBS**: Finkapen ez espezifikoa, **BB**: Finkapen basala.

%Inhibizioaren: 100-[(Inhibizioa-NBS)/(BB-NBS)x100]

**3.3 ekuazioa:** [$^{35}$S]GTPγS finkapena SPArekin konbinatzean lortutako emaitzen kalkulua finkapen basalarekiko portzentai izaera agonista, antagonista edo lehiakorra deskribatzeko. **NBS**: Finkapen ez espezifikoa, **BB**: Finkapen basala.

Kasu batzuetan, [$^{35}$S]GTPγSak Gα-proteina subtipo desberdinetara finkatzeko duen ahalmena aztertzeko, kontzentrazio-erantzunaren kurba osoa egin beharrean, kontzentrazio bakar batentzat (10 μM) azaldutako finkapena soilik aztertu zen. Kontzentrazio hori aukeratzerako orduan, finkapen maximoa

($E_{max}$/$I_{max}$) eragiteko ahalmena zuen farmakoaren kontzentrazioa hartu zen, kontzentrazio-erantzunaren kurbatik. Kasu horietan, finkapen basala %0 gisa adierazi zen, eta farmakoek eragindako efektua positibo edo negatibo gisa adierazi zen egoera basalarekiko.

Estimulazioaren edo inhibizioaren parametro farmakologikoen kalkulurako GraphPad Prism™ programa informatikoaren bidez lortutako analisi ez-lineala erabili zen. Horiekin, [$^{35}$S]GTPγSren finkapenarekiko efektu estimulatzaile eta inhibitzaile maximoak lortu ziren ($E_{max}$/$I_{max}$), baita horien erdiak lortzeko beharrezkoa den farmakoaren kontzentrazioak ere ($EC_{50}$/$IC_{50}$). Azterketetan lortutako puntu bakoitza **3.4** eta **3.5 ekuazioetan** sartu ziren kontzentrazio-erantzun kurba lortzeko. Eredu matematiko horrek onartutakoaren arabera, kontzentrazio-erantzunaren kurbaren malda estandarra da, Hillen maldaren balioa (edo malda faktorea) 1,0 izanik. Hori horrela, balio hori izan beharko litzateke malda, baldin eta masen ekintzaren legearen arabera, farmakoa hartzaile batera soilik finkatzen bada.

$$E= BB+(E_{max}-BB)(1+10^{(LogEC_{50}-Log[X])})$$

**3.4 ekuazioa**: Estimulazioaren kontzentrazio-erantzunaren kurba monofasikoa (malda estandarra duena). **E** erantzunari dagokio (3.2 ekuazioa estimulazio %), **X** kontzentrazioak eragindakoa**, BB** [$^{35}$S]GTPγSren finkapen basalari dagokio, agonista ezean lortutako finkapena (100% gisa adierazita), $\mathbf{E_{max}}$ efektu estimulatzaile maximoa (%) eta $\mathbf{LogEC_{50}}$ efektu estimulatzaile maximoaren erdia lortzeko beharrezkoa den farmakoaren kontzentrazioa.

$$E= BB+(I_{max}-BB)(1+10^{(LogIC_{50}-Log[X])})$$

**3.5 ekuazioa**: Estimulazioaren kontzentrazio-erantzunaren kurba monofasikoa (malda estandarra duena). **E** erantzunari dagokio (3.3 ekuazioa estimulazio %), **X** kontzentrazioak eragindakoa**, BB** [$^{35}$S]GTPγSren finkapen basalari dagokio, agonista ezean lortutako finkapena (100% gisa adierazita), $\mathbf{I_{max}}$ efektu estimulatzaile maximoa (%) eta $\mathbf{LogIC_{50}}$ efektu estimulatzaile maximoaren erdia lortzeko beharrezkoa den farmakoaren kontzentrazioa.

$E_{max}/I_{max}$ parametroak batezbestekoa±SEM (batezbestekoaren errore estandarra) gisa adierazten dira, eta $-LogEC_{50}/IC_{50}$ parametroak ere batezbestekoa±SEM gisa adierazten dira. Balio horiek izango dira analisi estatistikoa egiteko erabiliko direnak. Efektu estimulatzailearen edo inhibitzailearen maximoaren erdia lortzeko gai den farmakoaren kontzentrazioa $EC_{50}/IC_{50}$ gisa adierazten da. Parametro hori, $LogEC_{50}/IC_{50}$ balioen batezbestekoaren antilogaritmoa egitean lortzen da.

Hori guztiaz gain, kontzentrazio-erantzunaren kurbak ko-analisi bidez ere aztertu ziren (talde bererako egindako esperimentu guztien analisia aldi berean), talde bakoitzaren balio orokorrak lortzeko. Horretarako, [$^{35}$S]GTPγSaren finkapena leku bakar batera hobekien doitzen zuen erregresio ez-linealaren eredua erabili zen. Emaitzok, doikuntza onenaren balioa ± %95eko konfidantza-tartea gisa adierazi ziren.

## Analisi estatistikoa

Lortutako emaitzen interpretaziorako hainbat analisi estatistiko egin ziren. Hasteko, lortutako balio guztiak *Grubb's test* analisiaren bidez aztertu ziren, talde esperimental bakoitzean egon zitezkeen *outlier* balioak detektatu eta baztertzeko.

Kontzentrazio puntu bakarrarekin egindako esperimentuetan lortutako emaitzak lagin bakarrerako *Student's test* analisiaren bidez aztertu ziren;

farmakoak finkapen basalarengan eragindako efektua ala efektu-eza aztertzeko asmoz.

Bi buztanetako Student´s t-test parekatugabea erabili zen, bi egoera esperimental aztertu behar ziren bakoitzean; adibidez, agonistak eragindako efektua eta agonistak antagonistarekin batera eragindako efektuak alderatzeko. Hiru egoera desberdin alderatzeko, aldiz, bide bakarreko ANOVA analisia erabili zen, Bonferroniren *post-hoc* analisiarekin batera. Hori izan zen erabilitako analisia eskizofrenia taldea, eskizofrenia ez zuten suiziden taldea eta kontrol taldea alderatzeko, [$^{35}$S]GTPγSaren finkapenaren modulazioa aztertzerako orduan.

Bestalde, bi aldagaiek izan zezaketen eragina aztertzeko, bi bidetako ANOVA erabili zen, Bonferroniren *post-hoc* analisiarekin batera. Analisi hori izan zen egokiena genotipo desberdina zuten saguen taldeak alderatzeko, adibidez.

Aldagai independenteen (adina, PMD eta metatze denbora) eta menpekoak diren eragin funtzionalen arteko erlazioa aztertzeko Pearsonen r korrelazio koefizientea kalkulatu zen. Korrelazio hori esanguratsua zen kasuetan, kobariantza analisia (ANCOVA) egin zen talde esperimentalen artean (eskizofrenia, eskizofrenia ez zuten suizidak eta kontrolak). ANCOVA analisiak egiteko InVivoStat software estatistikoa erabili zen.

Lan honen emaitzak aztertzean, lortutako desberdintasunak estatistikoki esanguratsutzat jo ziren $p<0,05$ zela egiaztatu zenean.

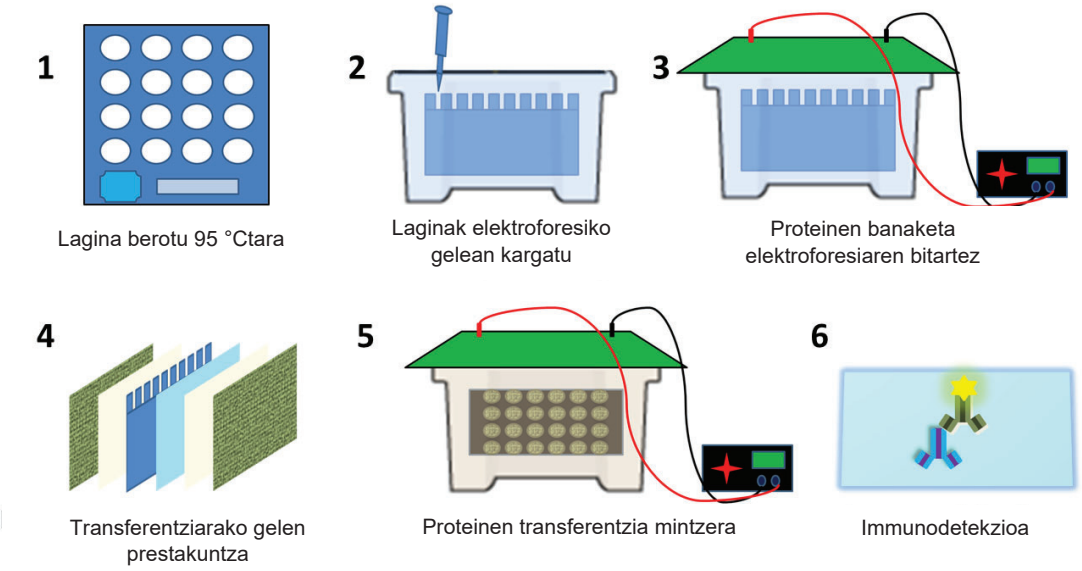
Hori guztiaz gain, analisi osagarriak ere egin ziren taldeen arteko desberdintasun potentzialak aztertzeko. Horrela, eskizofrenia, eskizofrenia ez zuten suizidak eta kontrol taldeko balioen koanalisian lortutako emaitza globalei, beste azterketa gehigarri bat egin zitzaien. (DeLean et al., 1978; Motulsky eta Ransnas, 1987) Analisi osagarri horretan, erabilitako analisi ereduen egokitasuna alderatu zen, behartze multzo bat aplikatuz F analisiaren bidez (Balioen karratuaren gehiketa printzipioan oinarrituta).

Hasteko, balio multzo bakoitza banaka aztertu zen (behartu gabe). Ondoren, balioen karratuaren emaitza lortzeko, doikuntza bakoitzean lortutako bakarkako balioen gehiketa egiten da, askatasun gradu kopuruarekin ere egiten den moduan. Ondoren, balio multzoak nahastuta ere aztertzen dira, aldi berean guztiak, eta amankomunak dituzten parametro bat, edo gehiago, partekatzera behartzen zaio doitutako kurbari (balio basalak, $E_{max}$/$I_{max}$ eta $LogEC_{50}$/$IC_{50}$). Horrela, balio ezberdinak lortzen dira balioen karratuekin eta askatasun graduekin. Parametro bat edo gehiago partekatzea baimentzen zuen analisia jo zen doikuntza egoki gisa, baldin eta bariantzen ondarra ez bazuen esanguratsuki handitzen. Egokitasunaren esaguratsutasun estatistikoa F test bidez neurtu zen, $p<0,05$ izanik, eta F[DFn, DFd] gisa adierazi zen; non F balioen banaketari dagokio, DFn zenbakitzaileko askatasun graduei dagokio eta DFd izendatzaileko askatasun graduei dagokio.

### 3.5.2 Western Blot frogak

Western Blot frogak luze eta zabal erabiliak izan dira proteinen detekzio eta identifikaziorako, antigorputz espezifikoak erabiliaz. Prozedura hainbat pausotan egiten da: lehenengo, elektroforesiaren bidez lagineko proteinak banatu egiten dira, pisu molekularraren arabera: ondoren, proteinak mugiezin bilakatu eta transferitu egiten dira, geletik nitrozelulosa mintzera, proteinak eskuragarri izanik antigorputzekin lotzeko. Azkenik, nitrozelulosa mintzak antigorputz espezifikoen disoluzioan sartzen dira, itu diren proteinak ezagutu eta horietara lotzeko.

Ikerlan honetan, lehenengo helburua, immunoprezipitazioarekin akoplaturiko [$^{35}$S]GTPγS finkapen teknikan (SPA) erabilitako antigorputzen karakterizazioa eta espezifikotasuna frogatzea izan zen, Gα-proteina azpimota bakoitzarentzat. Behin antigorputzak aztertuta, $G\alpha_{i1}$- eta $G\alpha_{q/11}$-proteinen dentsitate immunoerreaktiboa neurtu genuen postmortem aurre-garunazal laginetan eskizofrenia taldean, eskizofrenia ez zuten suizida taldean eta beraiekin parekatutako kontrol taldean. Era berean, zitoeskeletoaren zati den β-aktina proteina ere neurtu zen, jarritako proteina kopurua kontrolpean izateko asmoz.




**3.2 irudia**: Western Blot frogen adierazpen grafikoa. 1. Laginak 95ºCtan berotzen dira 5 minutuz; 2. Lagin kantitate optimizatua jartzen da gelean; 3. Gelen elektroforesia jartzen da abian 30 minutuz eta 60 V pean, lehenengo, eta ondoren 90 minutuz 140 V pean. Horrela, proteinen banaketa ematen da pisu molekularraren arabera, eta txikienek migrazio handiena jasango dute; 4. Transferentziarako sandwichak prestatzen dira; gela katodorantz jarririk (-) eta mintza anodorantz (+); 5. Negatiboki kargatutako proteinen migrazioa gertatzen da geletik mintzera, transferentzia 0,3 A pean eta 90 minutuz luzatzen da; 6. Mintzen finkapen ez-espezifikoa eragozten da esnea daraman Blocking tanpoia erabilita. Ondoren, mintza antigorputz primarioarekin inkubatzen da, eta, garbitu ostean, antigorputz sekundarioarekin inkubatzen da, ordubetez eta giro tenperaturan. Bukatzeko, irudien fluoreszentziaren neurketa egiten da. Irudia J. DelaCuesta-Barrutiaren tesirako sortutako ilustrazioa.

### 3.5.2.1 Mintzetan aberastutako laginen prestaketa ($P_2$ zatikia)

Western Blot frogak egiteko erabili ziren mintzen laginen prestaketa, SPA teknika egiteko erabili zirenen antzera egin zen (**3.5.1.1 atala**). Esperimentua egin zen egunean, 0,5 mg zituzten $P_2$ zatikia zuten pelletak desizoztu ostean, 125 µl Tris-HCl 0,5 nM tanpoian bereseki ziren. Lagin horiei, 119 µl 2X Laemmli lagin tanpoia eta β-mercaptoetanolaren 6 µl gehitu zitzaien, laginen proteina edukia 2 mg/ml izan zedin. Eskizofrenia zuten subjektuen,

eskizofrenia ez zuten suiziden eta kontrolen laginak aldi berean prozesatu ziren, egun berean.

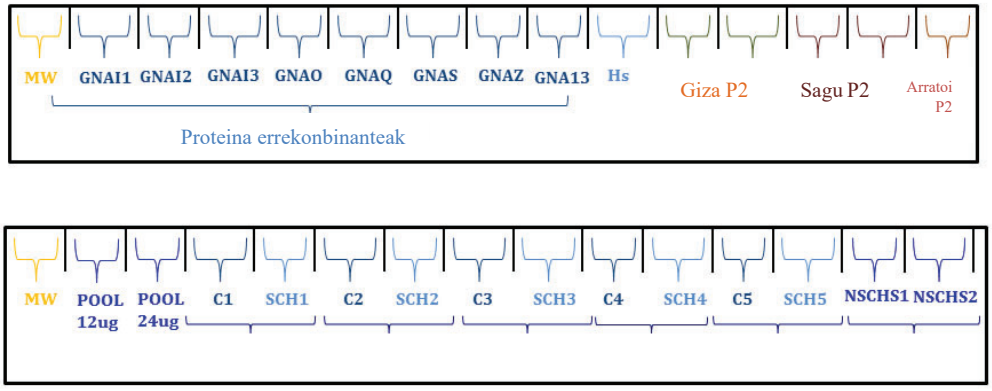
### 3.5.2.2 Gel elektroforesia, transferentzia eta immunodetekzioa

**Poliakrilamida geletan egindako elektroforesia**

Poliakrilamida geletan egiten den elektroforesia edo SDS-PAGE (*Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis*), desnaturalizazio egoeran egiten da, eta lagin bateko proteinen banaketa egiteko (pisu molekularraren arabera) gehien erabiltzen den teknika da.

Elektroforesian zehar, lehenengo, *stacking gel*ean migratzen dute proteinek. Gelaren zati horren osaketa %5 poliakrilamida zen, 125 mM Tris HCl, %0,1 SDS, 0,07% antigeno prostatiko espezifikoa (PSA) eta %0,14 TEMED zuen disoluzioan (pH 6,8). Laemmli-erreaktiboarekin prestatutako laginak 5 minutuz berotu ziren 95ºCtan Thermoblock gailuan, *stacking gel*a prestatzen zen (**3.2 irudia**). Laginean dauden proteinen banaketa, pisu molekularraren arabera, *runnig gel*era heltzen direnean hasten da. Gelaren zati horren osaketa %10 akrilamida-bisakrilamida zen 0,37 M Tris HCl, %0,1 SDS, %0,07 PSA eta %0,07 TEMED zuen disoluzioan (pH 8,8). Teflon™ orrazi bat sartu zitzaien gelei 15 hilara sortzeko, laginak jarriak ziren gelaren zatian.

Horrela lortutako, 15 hilaratan banatutako gelen neurriak 6 x 8 cm izan ziren (**3. irudia**). Hilara bakoitzean jarritako bolumena eta proteina kantitatea aztertu beharreko Gα-proteinaren araberakoa izan zen (**3.5 taula**). Gel bakoitzaren lehenengo hilaran pisu molekularraren markatzaile komertziala jarri zen (10-250 kDa, Precision Plus Protein™, Dual Color Standards, Bio-Rad). Gel bakoitzean jarraitu zen laginen eta antigorputzen karakterizazioan banaketa **3.3 irudia**n adierazita dago.




**3.3 irudia**: Gel bakoitzean jarritako laginen banaketa, antigorputzen karakterizazio esperimentuetarako goikoa eta taldeen arteko azterketa egiteko behekoa (eskizofrenia, eskizofrenia ez duten suizidak eta kontrolak). **MW**: Pisu molekularren markatzailea, **P2**: mintzetan aberastutako zatikien lagina, **C**: Kontrol subjektua, **SCH**: Eskizofrenia subjektua, **NSCHS**: Eskizofrenia ez duten subjektu suizida.

**3.5 taula**: Western Blot esperimentuetan erabilitako egoerak hainbat laginekin: gizakia, sagua eta arratoia. **MW** (Pisu molekularra).

| Proteina | Jarritako proteina/ bolumena | MW (kDa) | Blocking tanpoia | Inkubazio disoluzioa | Antigorputz primario diluzioa | Antigorputz sekundario diluzioa |
|---|---|---|---|---|---|---|
| **$G\alpha_{i1}$** | 30 µg/ 15 µl | 40 | %5 esne lehor gaingabetua | Blocking tanpoia + %0,1 Tween 20 | 1:200 | 1:10000 |
| **$G\alpha_{i2}$** | 15 µg/ 7,5 µl | 42 | %5 esne lehor gaingabetua | Blocking tanpoia + %0,1 Tween 20 | 1:300 | 1:8000 |
| **$G\alpha_{i3}$** | 15 µg/ 7,5 µl | 40 | %5 esne lehor gaingabetua | Blocking tanpoia + %0,1 Tween 20 | 1:300 | 1:8000 |
| **$G\alpha_{o}$** | 15 µg/ 7,5 µl | 45 | %5 esne lehor gaingabetua | Blocking tanpoia + %0,1 Tween 20 | 1:500 | 1:8000 |
| **$G\alpha_{q/11}$** | 24 µg/ 12 µl | 40 | %5 esne lehor gaingabetua | Blocking tanpoia + %0,1 Tween 20 | 1:300 | 1:8000 |
| **$G\alpha_{s}$** | 15 µg/ 7,5 µl | 41 | %5 esne lehor gaingabetua | Blocking tanpoia + %0,1 Tween 20 | 1:500 | 1:8000 |

Elektroforesia egiteko tanpoi berezia prestatu zen: 25 mM Tris HCl, 192 mM glizina eta %0,1 SDS, pH 8,3. Hasieran eta 30 minutuz 60 V aplikatu zitzaizkien gelei eta ondoren 140 V. Elektroforesia gelaren bukaera zatira heltzean bukatutzat jo zen, 90 minutu pasa ostean, gutxi gora behera (**3.2 irudia**).

**Nitrozelulosa mintzetara proteinen transferentzia**

Prozedura honetan, gelean aurkitzen diren proteinak nitrozelulosa mintzetara tranferitzen dira (poro tamaina: 0,45 µM) (GE Healthcare, Buckinghamsire, Erresuma Batua) transferentzia tanpoi bati esker: 25 mM Tris HCl, 192 mM glizina, %20 metanol, pH 8,3. Gelak eta nitrozelulosa mintzak zelulosazko paperekin batera estuki bildu ziren cassette berezi batzuetan, eta transferentzia izotzetan gertatu zen. Horretarako, 0,3 Atako eremu elektrikoa aplikatu zitzaien 90 minutuz. (**3.2 irudia**).

***Blocking* prozedura eta immunodetekzioa**

Behin transferentzia bukatuta, nitrozelulosa mintzak garbituak izan ziren, gehiegizko metanola kentzeko asmoz. Horretarako fosfatozko tanpoia erabili zen (PBS), (137 mM, NaCl 2,7 mM KCl, 12 mM $Na_2HPO_4$ eta 1,38 mM $KH_2PO_4$, pH 7,4). Ondoren, mintzak *bloking* disoluzioarekin batera inkubatu ziren ordubetez giro tenperaturan (%5 esne gaingabetu lehorra PBStan, pH 7,4), antigorputzen finkatze ez-espezifikoa ekiditeko eta *background* seinalea murrizteko. Denbora hori pasata, mintzak zegozkien antigorputz primario espezifikoaren diluzioarekin inkubatu ziren gau osoan zehar 4ºCtan (**3.5 taula**). Untxi jatorriko anti β-aktin antigorputza ere gehitu zitzaion disoluzioari 1:10000 diluzioan.

Hurrengo egunean, mintzak PBSaz garbituak izan ziren. Ondoren fluoreszentziaz markaturiko antigorputz sekundarioekin inkubatu ziren mintzak (Alexa Fluor® anti-sagua eta IRDye™ anti-untxia) (**3.5 taula**), inkubazio tanpoian disolbatua, ordubetez eta giro tenperaturan. Bukatzeko, nitrozelulosazko mintzak PBSaz garbitu ziren, gehiegizko antigorputz sekundario itsatsia kentzeko.

Antigorputz sekundario fluoreszentearekin inkubatutako mintzak detektatu eta kuantifikatzeko *Odyssey infrared imaging system* detektagailua erabili zen (LI-

COR Bioescience, Nebraska, EEBB) (**3.2 irudia**). IRDye™ 800 markatzailea zeraman antigorputza detektatzeko 800 nm uhin luzera erabili zen, berdean ikusten dena. Alexa Fluor® 680 markatzailea zeraman antigorputza detektatzeko, aldiz, 680 nm uhin luzera erabili zen, gorrian ikusteko.

Western Blot frogetan erabilitako antigorputzen espezifikotasuna egiaztatzeko Gα-proteina azpimota bakoitzeko antigorputz espezifikoa (**3.6 taula**) eta proteina errekonbinantea erabili ziren (**3.7 taula**). Horiek erabiltzean, Gα-proteina azpimoten arteko finkapen gurutzatua zehaztea lortu zen espezie bakoitzaren laginetan: gizakia, arratoia eta sagua.

**3.6 taula**: Western Blot eta SPA esperimentuetan erabilitako antigorputz monoklonalak (Ms: sagua, R: Arratoia, H: gizakia).

| Itua | Deskribapena | Produktu zenbakia | Saltsailea | M.W | Espezie erreaktibitatea | IgG azpimota | Azken kontzentrazioa |
|---|---|---|---|---|---|---|---|
| $G\alpha_{i1}$ | Saguaren anti-$G\alpha_{i1}$ monoklonala | sc-56536 | Santa Cruz | 41 kDa | Ms, R, H | Ms IgG2b | 0,1 µg/putzuko<br>9 ng/µg prot |
| $G\alpha_{i2}$ | Saguaren anti-$G\alpha_{i2}$ monoklonala | sc-13534 | Santa Cruz | 41 kDa | Ms, R, H | Ms IgG2b | 0,1 µg/putzuko<br>9 ng/µg prot |
| $G\alpha_{i3}$ | Saguaren anti-$G\alpha_{i3}$ monoklonala | sc-365422 | Santa Cruz | 45 kDa | Ms, R, H | Ms IgG3 | 0,033 µg/putzuko<br>3 ng/µg prot |
| $G\alpha_{o}$ | Saguaren anti-$G\alpha_{o}$ monoklonala | sc-393874 | Santa Cruz | 40 kDa | Ms, R, H | Ms IgG2a | 0,05 µg/ putzuko<br>4.5 ng/µg prot |
| $G\alpha_{q/11}$ | Saguaren anti-$G\alpha_{q/11}$ monoklonala | sc-515689 | Santa Cruz | 45 kDa | Ms, R, H | Ms IgG2b | 0,1 µg/ putzuko<br>9 ng/µg prot |
| $G\alpha_{s/olf}$ | Saguaren anti-$G\alpha_{s/olf}$ monoklonala | sc-377435 | Santa Cruz | 45 kDa/<br>52kDa | Ms, R, H | Ms IgG2a | 0,1 µg/ putzuko<br>9 ng/µg prot |

**3.7 taula**: Western Blot esperimentuetan erabilitako proteina errekonbinanteak

| Itua | Produktu zenbakia | Saltzailea | Pisu molekularra | Jarritako proteina | Lotea |
|---|---|---|---|---|---|
| GNAi1 | 32-3896 | Abeomics | 42.7 kDa + 23 aa HisTag | 100 ng | 413PGANI1 |
| GNAi2 | ABIN1355337 | Antibodies on-line | 42 kDa + GST Tag | 50 ng | H2141 |
| GNAi3 | 32-3898 | Abeomics | 43 kDa + 23 aa HisTag | 100 ng | 1114PGANI3 |
| GNAO1 | ABIN5709596 | Antibodies on-line | 40 kDa + 44 aa HisTag | 75 ng | 04055 |
| GNAq | ABIN1355345 | Antibodies on-line | 42 kDa + GST Tag | 75 ng | IC051 |
| GNAs | ABIN1355349 | Antibodies on-line | 47 kDa + GST Tag | 75 ng | HC141 |
| GNAz | CSB-EP009601HU | Cusabio | 56.8 kDa + 6xHis-SUMO | 75 ng | 03251 |
| GNA13 | CSB-EP618885HU | Cusabio | 48 kDa + 6xHisTag | 75 ng | 03257 |

### 3.5.2.3 Emaitzen analisi matematikoa eta estatistikoa

**Analisi matematikoa**

Antigorputz sekundariok sortutako fluoreszentzia detektatu eta kuantifikatzeko *Odyssey infrared imaging system* detektagailua erabili zen (LI-COR Bioescience, Nebraska, EEBB), eta horiek utzitako aztarnen dentsitatearen balio integratuak lortu ziren. Western Blot frogetan lortutako emaitzak erdi-kuantitiboak direla onartzen da, mintzetan aurkitzen den proteina kopurua zehaztea baimentzen duen arren, proteina estandarden kalibrazio zuzena izan gabe ezin delako proteinen kontzentrazio zehatza lortu.

Bestalde, geletan jarritako lagin bakoitzaren proteina kantitatea kontrolatzeko, β-aktina proteinaren immunoerreaktibitateaz zuzendu ziren lortutako dentsitateen balioak. Izan ere, aurretik frogatua zegoen zitoeskeletoaren β-aktina proteina ez dagoela aldatua ez eskizofrenia duten gaixoetan eta bere buruaz beste egiten duten suizidetan (Uriguen et al., 2009; Rivero et al., 2015). Gainera, esperimentuen artean ager daitekeen errore interesperimentala murrizteko asmoz, gel bakoitzean *pool* lagina ere aztertua izan zen. *Pool* laginak emandako balioa, beste laginek emandako balioa zuzentzeko erabilia izan zen, Western Blot esperimentu guztietan konstante mantentzen zelako. *Pool* laginak emandako balioari %100 balioa eman zitzaion ondorengo kalkuluak egiteko. Beste lagin guztien balioak lortutako immunoerreaktibitate erlatiboaren ehunekotan (%) adierazi ziren *pool* laginak sortutako seinalearekiko.

Subjektu bakoitzarentzat adierazitako balioa, egun eta gel desberdinetan lortutako emaitzen batezbesteko balioa da. Lortutako azken emaitza batezbestekoa±SEM gisa adierazita dago.

**Analisi estatistikoa**

Antigorputzen karakterizazioa egin zenean, lortutako dentsitateen balioekin ez zen inongo analisirik egin, helburua Gα-proteina azpimota bakoitzarekiko espezifikoak ziren ala ez aztertzea zelako. Hori horrela, pisu molekularraren arabera behar zen lekuan aztarnak ikusgai zeudenean soilik neurtu ziren.

Emaitza guztiak *Grubb´s test* analisiaren bidez aztertuak izan ziren, outlier baliorik zegoen aztertzeko, eta baiezko kasuetan analisian baztertzeko. Emaitzek banaketa gaussiarraren arabera banatuak zeuden ere aztertu zen kasu guztietan.

Gainera, kontrol taldea *One-sample Student's t-test* analisiaren bidez aztertu zen *pool* laginarekiko, beraien arteko desberdintasun esanguratsurik ez zegoela frogatzeko, %100 balioa lortzeko orduan.

Eskizofrenia taldea, eskizofrenia ez zuten suiziden taldea eta kontrol taldea alderatzeko bide bakarreko ANOVA analisia erabili zen, Bonferroniren *post-hoc* analisiarekin batera. Taldeen arteko emaitzak desberdinak zirela adierazi zen $p<0,05$ zen kasuetan.

Aldagai independenteen (adina, PMD eta metatze denbora) eta, GNAI1 eta GNAQ proteinen espresio mailen arteko erlazioa aztertzeko Pearsonen r korrelazio koefizientea kalkulatu zen. Korrelazio hori esanguratsua zen kasuetan, kobariantza analisia (ANCOVA) egin zen taldeen esperimentalen artean (eskizofrenia, eskizofrenia ez zuten suizidak eta kontrolak). ANCOVA analisiak egiteko InVivoStat software estatistikoa erabili zen (Mockett Media

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