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Interclass GPCR heteromerization affects localization and trafficking

A thesis submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

By

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Research articles:

de la Fuente Revenga M, Zhu B, Guevara CA, Naler LB, Saunders JM, Zhou Z, Toneatti R, Sierra S, Wolstenholme JT, Beardsley PM, Huntley GW, Lu C, González-Maeso J. Prolonged epigenomic and synaptic plasticity alterations following single exposure to a psychedelic in mice. *Cell Rep.* 2021 Oct 19;37(3):109836

Sánchez-González A, Thougard E, Tapias-Espinosa C, Cañete T, Sampedro-Viana D, Saunders JM, Toneatti R, Tobeña A, González-Maeso J, Aznar S, Fernández-Teruel A. Increased thin-spine density in frontal cortex pyramidal neurons in a genetic rat model of schizophrenia-relevant features. *Eur Neuropsychopharmacol.* 2021 Mar;44:79-91

Shah UH, Toneatti R, Gaitonde SA, Shin JM, González-Maeso J. Mapping the interface of an heteromeric GPCR complex using genetically encoded photo-crosslinkers in living cells *Cell Chem Biol.* 2020 Oct 15;27(10):1308-1317.

Toneatti R, Shin JM, Shah UH, Saunders JM, Mayer CR, Janssen W, Conway DE, Bensson DL, González-Maeso J. Interclass GPCR heteromerization affects localization and trafficking, *Science Signaling* 2020 Oct 20;13(654)

de la Fuente Revenga M, Ibi D, Cuddy T, Toneatti R, Kurita M, Ijaz MK, Miles MF, Wolstenholme JT, González-Maeso J. Chronic clozapine treatment restrains via HDAC2 the performance of mGlu2/3 agonism in a rodent model of antipsychotic activity. *Neuropsychopharmacology* 44:443-454 (2019)

de la Fuente Revenga M, Ibi D, Saunders JM, Cuddy T, Ijaz MK, Toneatti R, Kurita M, Holloway T, Shen L, Seto J, Dozmorov MG, González-Maeso J. HDAC2-dependent antipsychotic-like effects of chronic treatment with the HDAC inhibitor SAHA in mice. *Neuroscience* 388:102-117 (2018)

Book chapter:

Salvador Sierra, Rudy Toneatti, Javier González-Maeso,
Chapter 8 - Class A GPCR oligomerization: Reasons of controversy,
Editors: Beata Jastrzebska, Paul S.-H. Park,
GPCRs. Structure, Function, and Drug Discovery
Academic Press, 2020, Pages 121-140.

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LIST OF ABBREVIATIONS

5-HIAA: 5-hydroxy indole acetic acid	GPCR: G protein-coupled receptor
5-HT: 5-hydroxytryptamin	GRK: G protein-coupled receptor kinases
7TMR: Seven transmembrane receptors	HAL: Hallucinogenic/Psychedelic compound
AAP: Atypical antipsychotic	ICD: Intracellular domain
AP-2: Adapter protein-2 complex	iGluR: Ionotropic glutamate receptor
β_2 AR: β_2 -adrenergic receptor	ISH: <i>in situ</i> hybridization
BBB: Blood brain barrier	IP: Inositol phosphate
BDS: Bright detail similarity	IP3: Inositol triphosphate
BLOC-1: Biogenesis of Lysosome-related Organelles Complex-1	κ OR: κ -opioid receptor
BRET: Bioluminescence resonance energy transfer	LE: Late endosome
cAMP: Cyclic adenosine monophosphate	LSD: Lysergic acid diethylamide
CPN: Caudate-putamen nucleus	mGluR: Metabotropic glutamate receptor
CSF: Cerebrospinal fluid	MAPK: Mitogen-activated protein kinase
CoIP: Coimmunoprecipitation	MAO: Monoamine oxidase
DAG: Diacylglycerol	MVB: Multi-vesicular body
DAMGO: [D-Ala ² , NMe-Phe ⁴ , Gly-ol ⁵]-enkephalin	μ OR: μ -opioid receptor
DPDPE: [D-Pen ^{2,5}]Enkephalin, [D-Pen ² , D-Pen ⁵]Enkephalin	NAC: Nucleus accumbens
DOI: (\pm)-2,5-Dimethoxy-4-iodoamphetamine	NAM: Negative allosteric modulator
DMT: Dimethyltryptamine	NHAL: Non hallucinogenic/ non Psychedelic compound
DxR: Dopamine receptors x	PAM: Positive allosteric modulator
ECD: Extracellular domain	PFC: Prefrontal cortex
EE: Early endosome	PI3K: Phosphatidylinositol 3 kinase
EEA: Early endosome antigen	PIP2: Phosphatidyl inositol-bisphosphate
EEAT: Excitatory amino acid transporter	PKA: Protein kinase A
ER: Endoplasmic reticulum	PKC: Protein kinase C
ERK: Extracellular signal-regulated protein kinase	PLA: Proximity ligation assay
δ OR: δ -opioid receptor	PLC: Phospholipase C
FRET: Förster resonance energy transfer	PSD: Post synaptic density/PSD-95
GEF: Guanosine nucleotide exchange factor	RE: Recycling endosome
GA: Golgi apparatus	RTK: Receptor tyrosine kinase
GABA _B R: gamma-aminobutyric acid receptor type B	RRI: Receptor-receptor interaction
GAP: GTPase activating proteins	SE: Sorting endosome
GDP: Guanosine diphosphate	SERT: Serotonin reuptake transporter
GIRK: G protein-activated inwardly rectifying K ⁺ channel	SNARE: Soluble NSF attachment protein receptor
GTP: Guanosine triphosphate	TM: Transmembrane (domain)
	VGLUT-1: Vesicular glutamate transporter

STATEMENT OF CONTRIBUTIONS

Figure 6 J has been generated by **Dr. M. Fribourg**.

Figure 5 A, B and C, and Figure A1 have been generated by a Python software manipulated by **Dr. Carl R. Mayer** who trained me to use it.

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Jong M Shin helped me for cell lines characterization binding assays.

This story is dedicated to my Father (February 1933 – October 2020)

Information is any difference that makes a difference

Gregory Bateson

I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery has its own beauty.

Marie Curie

The wonder of nature does not become smaller because one cannot measure it by the standards of human moral and human aims.

Albert Einstein

My life seemed to be a series of events and accidents. Yet when I look back, I see a pattern.

Benoît B. Mandelbrot

ABSTRACT

Class A serotonin (5-hydroxytryptamine) 2A (5-HT_{2A}R) and class C metabotropic glutamate 2 receptors (mGluR2) are seven transmembrane receptors (7TMRs or G protein-coupled receptors – GPCRs) involved in multiple neuropsychiatric disorders including schizophrenia. Previous findings from our laboratory reported that 5-HT_{2A}R and mGluR2 are dysregulated in the prefrontal cortex of patients suffering from this psychiatric condition, although 5-HT_{2A}R's expression was recovered in antipsychotic-medicated patients. Genome-wide association studies on schizophrenia reported that endosomal trafficking that regulates cell surface abundance of another 7TMR implicated in this disease (dopamine D2 receptor) can be altered. Ligand-activated receptors, including the 7TMR superfamily, are dynamic entities whose signaling depends on trafficking, and trafficking on signaling. Impairments in the spatial and temporal regulation of 7TMRs result in homeostasis perturbations and later in diseases. Like other cell surface proteins expressed in mammals, such as enzyme-associated receptors and ligand-gated ion channels, 7TMRs form homo- or hetero-oligomers, conferring novel and unique functional properties to the complex. Our previous findings suggested that 5-HT_{2A}R and mGluR2 are assembled in a functional GPCR heteromeric complex in both heterologous expression systems and in mouse and human brain frontal cortex. However, evidence that GPCR heteromerization can directly affect receptor subcellular organization and localization is missing, especially across classes. Here, we showed in mammalian HEK293 cells that 5-HT_{2A}R affected the localization and trafficking of mGluR2 through a mechanism that required their physical assembly. In the absence of agonists, 5-HT_{2A}R was primarily localized within intracellular compartments. Intriguingly, the plasma membrane-localized mGluR2 was redistributed in internal compartments when coexpressed with 5-HT_{2A}R. Agonists for either 5-HT_{2A}R or mGluR2 differentially affected trafficking through Rab5- or Rab7-positive endosomes in cells expressing the receptors alone, or together. In addition, overnight blockade of 5-HT_{2A}R with antipsychotic clozapine, but not with M100907, decreased mGluR2 density through a mechanism that involved 5-HT_{2A}R-mGluR2 heteromerization. Using TAT-tagged interfering peptides and a chimeric construct unable to heteromerize with 5-HT_{2A}R, we showed that heteromerization was necessary for the 5-HT_{2A}R-mediated co-trafficking of mGluR2. Furthermore, using bimolecular fluorescence complementation (BiFC), we tracked the maturation of the 5-HT_{2A}R-mGluR2 complex in the endoplasmic reticulum and the Golgi apparatus. In accordance with our findings in HEK293 cells, mGluR2/Rab5 colocalization was diminished in neuronal cortical primary culture of 5-HT_{2A}R^{-/-} mice as compared to controls. Additionally, mGluR2 was mostly localized near the plasmalemma in brain sections of 5-HT_{2A}R^{-/-} mice frontal cortex glutamatergic excitatory postsynaptic compartments. Together, our data suggest that GPCR heteromerization may itself represent a mechanism of receptor trafficking and sorting.

Introduction

7TMRs superfamily

With more than 800 members (a number that varies across studies) counting for about 2-3% of the total human protein-encoding transcripts¹⁻³, guanine nucleotide binding protein (G protein)-coupled receptors (GPCRs), or more accurately, heptahelical or seven transmembrane receptors (7TMRs), constitute the largest superfamily of spanning membrane proteins in the Metazoa and the mammalian genome. Mammals express about 400 non-odorant 7TMRs⁴. As a matter of comparison, the second largest family of receptors, the receptors-enzyme tyrosine kinase (RTK) family counts about 58 receptor-types in 20 subfamilies⁵. Because they represent fundamental interfaces between a tremendous diversity of extracellular stimuli*, triggering inner molecular cascades, cellular responses and genes transcription, 7TMRs provide highly valuable targets for clinical pharmacology. About 34% of FDA approved drugs target 107 unique 7TMRs, of which 27% treat neuropsychiatric disorders^{6,7}. Often termed metabotropic receptors, as opposed to ionotropic receptors, whose activation and subsequent cell reaction is faster than the former, they process and transduce signals throughout structural and biochemical steps much slower than receptor channels. A cell response is always a combination and a cooperation between fast and slow transmitters. The classical/canonical mode of signal transduction through 7TMRs cognate G proteins is widely accepted, although recent findings proposed G protein independent signaling⁸⁻¹⁰. For a concern of accuracy over widely acceptance, this report will use the term 7TMRs.

7TMRs and G protein signaling likely appeared before Plants, Fungi and Animals, and diverged from their common ancestor about 1.2 billion years ago¹¹. Primitive structures of 7TMRs existed in unicellular eukaryotes that resemble Metazoa cells¹². The glutamate-receptor-like receptor is one of the oldest. The serotonin receptors subfamily is also one of the oldest in class A/rhodopsin-

* Stimuli can be exogenous chemical ligands such as drugs, vitamins, odorants, tastants, and viruses, and physical stimuli such as photons. Endogenous ligands can be biogenic amines, nucleotides, hormones, large and small peptides, cations and lipids, small molecule neurotransmitters, chemokines, and more.

like 7TMRs, which appeared between 700 and 800 million years ago¹³. This evolutionary success gives an idea of the robustness of such biostructures, having evolved and diversified under selective pressure, to finally regulate almost all aspects of living organisms' physiology. 7TMRs share a minimal and unique common attribute that is the seven transmembrane α -helices domain connected by three extracellular (EC1, EC2, EC3) and three intracellular (IC1, IC2, IC3) alternated loops, in between an extracellular N-terminus and an intracellular C-terminus, often ended by a so-called helix 8.

Classification of 7TMRs

All characterized 7TMRs, as well as the orphan 7TMRs, are classified and categorized within the International Union of Basic and Clinical Pharmacology (IUPHAR) database[†], which recognizes 6 classes, widely used among studies. Several other classifications are often based on structural homology, but not only. 7TMRs are organized into 5 or 6 major nomenclatures according to their sequence similarity and functional properties (A to F)¹⁴, as well as structural and ligand-binding features (1 to 5)¹⁵. Also referenced phylogenetically as GRAFS¹⁶, acronym for Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin, the rhodopsin-like or class A/1 is the most represented subfamily in the human genome. The class A includes, with light-activated rhodopsin and highly numerous olfactory receptors, monoaminergic norepinephrine, dopamine and serotonin neurotransmitters/modulators transducers. Their homology of sequence indicates that they also share palmitoylated cysteine at their C-terminal tail, as well as a triplet of glutamic/aspartic acids - arginine/tyrosine termed E/DRY motif on their helix III¹⁷. This motif is the target of a strong ionic interaction that is involved in receptor activation as shown for the 5-HT_{2A} receptor, thought to be a generalizable activation system within the class A¹⁸. Another shared feature of class A receptors is that they harbor a long N-terminus with conserved cysteines¹⁷. Class B/2 are secretin-like 7TMRs among which corticotrophin-releasing factor (CRFR1, CRFR2),

[†] www.guidetopharmacology.org/GRAC/GPCRListForward?class=A

parathyroid hormone (PTH1R, PTH2R) and glucagon-like peptide receptors (GLPR) among others may be mentioned. Class C/3 regroups metabotropic glutamate (mGluR) and gamma-aminobutyric acid type B receptors (GABA_BR), which bind respectively the main excitatory/inhibitory neurotransmitters in the central nervous system (CNS), along with the sweet and umami taste receptors (T1R1/2+3) and the calcium sensing receptors (CaSR). Classes D and E constitute two minor families not represented in vertebrates, like cAMP receptor involved in protozoan chemotaxis signaling, and class F includes frizzled/smoothed receptors¹⁹. Further analogies between those nomenclatures remains unlikely since the criteria used are not conform.

Signal transduction

External ligands or stimuli are typically treated in two ways. Ionotropic receptors convey fast information flux, whereas metabotropic receptors transmit signals slowly, engaging a series of chemical and/or physical modifications from ligand binding to cell response. The signal transduction can be simply represented as an information crossing a regulated field. Information, the ligand, is treated, sorted and processed by the transmitter and its cognate G proteins, together with intracellular signaling molecules. A series of phosphorylations takes place within cascades, where the signal can be amplified and/or transmitted to the different target components. Because cell's phospholipid bilayer membrane must maintain the system closed and tightly regulated, communications operate uniquely through transmitters and channels, or both, and by endo/exocytic vesicular trafficking. Nonetheless, it is likely that other modes exist (gasotransmitters²⁰).

Receptor activation starts with ligand binding that initiates conformational changes within the seven transmembrane domains that resonate to receptor extremities, and convey signal transduction through heterotrimeric $\alpha\beta\gamma$ G proteins' subunits. The inactive state, reflecting basal activity, remains stable during low ligand concentration. Upon binding of its ligand, receptor changes its conformation that activates its cognate G protein. The activation/deactivation process

should not be envisioned as an on/off switch, with two distinct conformations. Receptors are in constant and continuous vibration, oscillating between a multitude of different conformations, toward active or inactive state, and get stabilized by the ligand²¹. It is considered as a multi-active state model²².

The specificity of the GPCR-mediated signal transduction was initially thought to solely lay in the dissociation of the trimeric G protein's $\alpha\beta\gamma$ subunits upon ligand binding. The α subunit catalyzes GDP to GTP to exchange and split from the tripartite GTPase to signal downstream effectors and second messengers, while $\beta\gamma$ is involved in regulating ions channels switching, as well as other effector proteins. The GTPase family of G proteins is also well diversified, the four main G proteins being $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. The first two respectively stimulate or reduce adenylyl cyclase activity, resulting in the augmentation or the reduction of cyclic adenosine monophosphate (cAMP) internal concentration. The cAMP-dependent protein kinase A (PKA) signal is amplified or dampened accordingly, which modulates specific cell responses. A typical example is the human class A $G\alpha_s$ -coupled β_2 adrenergic receptor ($\beta_{2A}R$), which triggers cardiac myocytes contraction after binding of adrenaline in tissues involved in sympathetic neurotransmission²⁶. Class C mGluR2/3 glutamatergic receptors are linked to the $G\alpha_{i/o}$ pathway that inhibits cAMP production and reduces glutamatergic tone in presynaptic terminals where it acts as an autoreceptor. The class A 5-HT_{2A} receptor provides an example of $G\alpha_{q/11}$ coupling. Agonist binding stabilizes the receptor in its active conformation that triggers the phospholipase C (PLC) DAG/IP3/PKC pathway. For example, in 5-HT_{2A}R-expressing neurons, the $G\alpha_q$ activates PLC- β which catalyzes the hydrolysis of phosphatidyl inositol-bisphosphate (PI(4,5)P2) into two second messengers, diacylglycerol (DAG) and inositol triphosphate (IP3). DAG remains attached to the membrane and recruits the protein kinase C (PKC), while IP3 diffuses in the cytoplasm to trigger calcium release from the endoplasmic reticulum storage. While diffused into the cytoplasm, Ca^{2+} can operate several cellular events such as activation of PKC (feedback), stimulation of ionic channels and

enzymes recruitment that together modulate synaptic transmission. The PKC is also involved in triggering cellular responses, such as neuronal plasticity, cell survival, and transcription factors activation. Several studies recently showed that the canonical linear model was not sufficient to describe unexpected results while tracking G protein activation. This led to separate G protein-dependent and -independent signaling^{8-10,23-26}.

To prevent response overload, and/or to return to basal state, *i.e.* low ligand concentration, a termination mechanism shuts down the signaling components by acting directly on the receptor, typically involving two major pathways: the receptor phosphorylation by G protein-coupled receptor kinases (GRK), and the subsequent recruitment of β -arrestins (1 or 2). GRK are recruited by G $\beta\gamma$ once released upon ligand-dependent receptor activation and split from the α subunit, so that receptor phosphorylation happens relatively fast after ligand binding. β -arrestins are thought to specifically bind GRK-phosphorylated receptors while competing with the G proteins in the vicinity. Arrestins are responsible for the recruitment of the endocytic machinery that involves dynamin and the formation of clathrin-coated vesicles. Successive intracellular mechanisms such as Rab-dependent desensitization (receptor endocytosis) and recycling from endosomes further affect receptor's fate and cell's responsiveness.

7TMRs structure and oligomerization

Class A 7TMRs or rhodopsin-like receptors share the minimal features that defines the superfamily, a seven transmembrane (TM) spanning helices domain, or heptahelical domain, connected by three intracellular loops (IL1-3) and three extracellular loops (EL1-3). This class of receptors has an extracellular cavity within their TM domain dedicated to the binding of the orthosteric ligand. They have also in common few conserved motifs²⁷. They are devoid of the large extracellular bilobal domain (ECD) characteristic of the class C 7TMRs, the Venus Fly Trap (VFT) domain. The typical receptors of this family, the GABA_B and the metabotropic glutamate receptors (mGluRs), share this VFT domain dedicated to the binding of the orthosteric ligand, as well as

holding a cysteine disulfide bridge necessary for the receptors obligatory dimerization. A cysteine rich domain is also partially involved in the dimeric interface, and proposed to relay ligand-induced conformational adjustment²⁸. The lack of covalent bond between protomers have led some reports to justify that class A dimers are not stable enough to remain bound supported by weak interactions²⁹. Class A receptor dimers are indeed less stable than class C^{30,31}. This does not rule out that they are functional. The rapid conversion between monomers and dimers and their resulting ratio may regulate receptor function³⁰.

Since the first biochemical findings showing that a peptide derived from a β_2 adrenergic receptor (β_2 -AR) transmembrane (TM) domain prevented both receptor complex formation and agonist-promoted stimulation of adenylyl cyclase activity³², the class A 7TMRs never ceased to attract attention for their ability to associate as dimers or higher oligomers^{33,34}. Despite certain limitations and caveats of most biophysical and biochemical approaches and technologies, including coimmunoprecipitation (CoIP), crosslinking, bioluminescence and Förster resonance energy transfer (BRET/FRET), SNAP- or CLIP-tag^{35,36}, bimolecular fluorescence complementation (BiFC) and other protein complementation assays (PCA), these studies generally provide evidence related to the fundamental role of class A 7TMR homo/heteromerization in the control and regulation of both agonist-induced receptor-G protein coupling^{37,38} and trafficking³⁹⁻⁴³. Similarly, contemporary advanced techniques such as single molecule imaging in intact cells indicate that certain class A 7TMRs form stable homomers^{41,44-46}, while others, using similar approaches, have shown either transient interactions^{30,47,48}, or negligible to absence of class A dimerization⁴⁹⁻⁵¹. Accordingly, many questions still remain open about the role of receptor complex formation in 7TMR's dynamics and behavior.

Between 2000 and 2004, a series of articles published in the Journal of Biological Chemistry (JBC) by several laboratories either directly implied the oligomeric association as a mean for 7TMRs to undergo internalization after ligand binding or assumed that receptor oligomerization could

influence both trafficking and signaling. These results directly followed pioneer discoveries about both class A and class C 7TMRs with the case of β_2 -AR and GABA_BR, respectively (see below). Among those studies, the scope has been extended to several other subtypes, β_2 -AR heterodimerization with β_1 -AR⁵²⁻⁵⁴, β_2 -AR with β_3 -AR⁵⁵, oligomerization of other adrenergic subtypes alpha, such as α_{1a} - and α_{1b} -AR⁵⁶, but also across subtypes⁵⁷. Likewise, an early study demonstrated that the δ -opioid receptor dimerize, suggesting that such receptor-receptor-interaction (RRI) was particularly important for their desensitization and subsequent internalization^{58,59}. Dimerization was also found to play a critical role in the agonist-independent desensitization and internalization of the human platelet-activating factor receptor (hPAFR)⁶⁰. These novel ideas also emerged within the opioid receptor subfamily following a series of impactful discoveries, such as the oligomerization of the μ - and the δ -opioid receptors⁶¹, where again, the scope extended the idea that those associations, this time among different protomer subtypes, would create novel functional properties that was not previously observed by classical pharmacological characterization. In the same journal, another study implicated heteromerization of somatostatin and μ -opioid receptors as a principal component of the cross-phosphorylation and the desensitization of both receptors⁶².

Although few studies directly implicated the 7TMRs homo/heteromerization in both endocytosis and biosynthesis processes, several of them though clearly suggested that such interactions can modulate function and trafficking. Opioid receptors were the first class A 7TMR to be thoroughly studied following the discoveries of dimerization and oligomerization within the subfamily. The μ - and the δ -opioid receptors have been demonstrated to heterodimerize using epitope tagged constructs submitted to subsequent immunoprecipitation. Moreover, whereas DAMGO (μ selective agonist) did not affect desensitization of the receptors expressed together, treatment with DPDPE (δ selective agonist) induced a resistance to desensitization and internalization of the two receptors. Opioid receptors are well studied for their capacity to be internalized and

desensitized after ligand binding. The fact that this process was affected by the coexpression and the putative interaction between μ and δ suggested that heteromerization can also affect basic components of class A receptors function such as internalization and desensitization⁶¹, as well as binding affinities of the ligands⁶³. These two studies published the same year demonstrated that oligomerization is an important feature of the opioid receptor subfamily. Heterodimerization also was demonstrated to create novel functions for the δ - and the κ -opioid receptors⁶⁴. Although the three opioid transmitters share a relatively high homology of sequence (about 65% identical amino acids), the diversity of RRI demonstrates a great variety of novel functions that affect most aspects of receptor signaling and trafficking. Together these pioneer studies provided the first indirect evidence that one protomer is able to affect both signaling and trafficking of another protomer within the class they belong to.

Within the class C, aside from similar conclusions about the novel properties provided by receptor homo/heteromerization, it has been shown that such associations are necessary for the proper cell surface addressing of the protomers together, as a single entity. The classical case illustrated by the class C GABA_BR remains remarkable as it is considered a paradigm. This obligate dimer is composed by the GABA_BR1 protomer that is unable to trigger G protein-activated inwardly rectifying K⁺ channels (GIRKs) signals alone, when overexpressed in mammalian cells: it requires heterodimerization with GABA_BR2, a subsequently described protomer that shares 35% homology with the subtype R1⁶⁵. The reason behind this resides in the early association of the two subunits; while crossing the endoplasmic reticulum during the protomers biosynthesis, R2 masks a retention motif lodged at the R1 C-terminus^{66,67}, allowing the full heteromer to exit the ER, otherwise retained in the organelle as single units. Here, heterodimerization is necessary for the cell surface addressing of a functional GABA_BR. These findings also indicated that the GABA_B receptor is an obligate dimer. More recently, a group demonstrated that GABA_BR2 competes with a gatekeeper protein termed PRAF2, which results in passing prerequisite quality controls prior to ER exit⁶⁸. It

highlights that 7TMRs can hold the role of chaperonin-like proteins, although finely regulated by resident chaperone molecules⁶⁹⁻⁷¹. Similarly, the class A β_2 -AR homodimerizes in the ER to allow upward trafficking to the plasma membrane. Using two different mutation approaches, it was revealed that retention/export motifs mutants act as dominant negative on WT β_2 -AR species, precluding their ER export and migration to the cell surface: first, by generating a chimera where the C-terminus tail of the β_2 -AR is interchanged with that of GABA_BR1, containing the above-mentioned retention motif; second, by mutating an ER export signal from the D₁R sequence in order to generate an unrecognizable binding site for specific resident proteins involved in the ER exit of D₁R. The structure/function role of such coupling has also been illustrated by the required dimerization of metabotropic glutamate receptor 2 to enable ligand-induced G protein activation, while monomeric 7TM domain appears sufficient for its coupling⁷², supporting the idea that dimers are the minimal signaling units, constituting a 2:1 ratio with the G protein they are associated to⁷⁰. Although truncated mGluR2 lacking their ECD can homodimerize using only the 7TM interface, the stability of this formation is assured by the cysteine 121 located in the VFT that provides a covalent intersubunit disulfide bond. Isolation of truncated receptors (purified 7TM domains) in lipid nanodiscs producing two different fractions enriched with two or one receptor, mGluR2 was able to signal with the latter fraction using positive allosteric modulator (PAM), which binds the ECD, but not the VFT. However, truncated mGluR2 lacking the VFT domain were unable to activate the G protein in presence of the orthosteric ligand glutamate, supporting the fact that only full length dimeric species can activate G α_i protein signaling as assessed by GTP γ S assay, while monomeric species are sufficient to the coupling to the G protein⁷³.

The accurate description of the interactions between residues that hold weak forces and stabilize oligomeric interfaces is an essential component of the pharmacological properties of 7TMRs, besides providing important information for the development of therapeutics and 3D models, which simulate dynamic structures and interactions at the atomic level. Advances in structural biology

technologies have allowed to elucidate and validate the precise transmembrane domains and often the residues involved in the conformations responsible for the activation/deactivation of oligomeric receptors. Across studies, TM domains I, IV and V typically appear to be the implicated interfaces in the formation of transient and/or stable class A 7TMRs oligomers⁷⁴.

How TM domains associate is well illustrated by the class C metabotropic glutamate receptors, which exhibit the most stable RRI within the superfamily. It was first proposed that the mGlu5 receptor is a naturally occurring dimer linked by a disulfide bond at the extracellular domain, and that concept could be generalizable to all mGluRs⁷⁵. The prototypic class A dimerization held by the μ -opioid receptor has been challenged, suggesting that the dimer populations are scarce and negligible at physiological concentrations⁵¹. Nevertheless, a great majority of theoretical approaches remain in favor of receptor oligomerization that provides an array of supplementary functions: antagonistic assemblies to cross-modulate protomer signaling, chaperones and guides through the biosynthesis pathway, subcellular co-trafficking and co-desensitization: dynamic or stable, kiss-and-fly or covalent^{76,77}.

In a review focused on class A 7TMRs homo/heterodimerization, several studies are listed according to the technique used and the involvement of the TM interaction to stabilize the dimeric formation, seven of nine class A receptor homo/heterodimers involve at least the TM IV and/or TM V. In a second chart similar findings are proposed⁷⁸. The diversity of the TM interaction is provided by the highly flexible linkers and loops that allow fast conformational modifications and relatively large movements of the TM domain^{78,79}. TM I and II were also mentioned for the muscarinic receptor 3 (M3), suggesting that each combination of the transmembrane interfaces is specific to a mode of functioning, either active or inactive states⁷⁴. At the time of this report, a study elucidated the active and inactive conformation used by the mGluR2 dimer to activate $G\alpha_i$ protein using cryoEM technology, showing asymmetric positioning of the TM helices both during activation and at basal inactive state, involving TM III/IV, and TM VI/VI respectively⁸⁰.

As illustrated by the muscarinic acetylcholine receptor 2 (M₂R) case, which has been found as monomeric, but also in dimeric and tetrameric forms by three different studies using different techniques and systems, class A 7TMRs could associate in various homomeric ratios^{74,81–83}. Although discrepancies for the same target receptor may be interpreted as a lack of reproducibility, feeding the debate on class A 7TMRs oligomerization, it might instead indicate that each study has revealed a different state of this receptor at a different time and under different conditions, as has been noted,

“Until recently, these were often considered to be mutually exclusive scenarios, but the co-existence and potential interchange between such forms, based in part simply upon mass action, has resulted in a more textured view.”⁸⁴

Receptor oligomers can be formed by different mechanisms, by weak forces at the plasma membrane, or covalently during biosynthesis. The origin of the coupling must have a physiological significance^{85–87}. Homo or heterodimerization most likely occurs within the same 7TMRs classes due to more compatible structural homology of their TM domain^{15,70}, the natural interface of receptor coupling. Nevertheless, interclass receptor associations are progressively more reported⁸⁶. A remarkable association has yet been suggested for the 5-HT₂R and the CRFR1⁸⁸. In this proposed model, 5-HT₂R cell surface expression and signaling is increased by agonist-stimulated CRFR1 trafficking. The accuracy in detecting the physical interaction at the interface of TM dimerization starts to provide more granular visualization on how oligomers associate and function⁸⁹. The proof provided by visual evidence is also a criteria in favor of 7TMRs oligomerization⁹⁰. Finally, the theory of moonlighting proteins, as a powerful and economic natural feature of proteins to organize, associate and function, support the idea that (receptor) oligomerization is not a rare and fortuitous event, but rather a common mechanism found everywhere that enables complexity and diversity^{91–93}.

Serotonin

Serotonin is the first neurotransmitter/trophic factor to appear in both Protozoan and Metazoan⁹⁴, probably inherited from unicellular eukaryotic precursors, which likely firstly evolved in mitochondria a billion years ago⁹⁵. Plants produce a large amount of serotonin for both trophic factor that regulates growth together with auxin and antioxidants to preserve cell from damages provoked by excessive production of molecular oxygen⁹⁶. Serotonin-based analogs and alkaloids such as ibogaine, mescaline, dimethyltryptamine (DMT) and psilocybin (in the Fungi) are produced by plants and fungi to resolve the problem of oxidation and protect them from radical oxygen, which is intrinsic to their photosynthetic abilities⁹⁵. Serotonin is a biogenic amine synthesized from indole precursor tryptophan primarily after its hydroxylation into 5-hydroxytryptophan by the tryptophan hydroxylase enzyme. Its product subsequently undergoes a decarboxylation by a decarboxylase enzyme in presence of the cofactor pyridoxal phosphate which produces 5-hydroxytryptamin (5-HT). Serotonin does not cross the blood brain barrier (BBB) but L-tryptophan does, being the rate-limiting precursor for the synthesis of serotonin. After being released presynaptically in the extracellular milieu (synaptic cleft), serotonin is either metabolized by monoamine oxidase (MAO) into 5-hydroxy indole acetic acid (5-HIAA), or recycled by serotonin reuptake transporter (SERT) or autoreceptors. 5-HIAA is an interesting metabolite that could be used as a marker because of its suggested abnormal levels in psychiatric disorders (often found as decreased), reflecting serotonin levels in the brain⁹⁷. Altered blood, serum or cerebrospinal fluid (CSF) levels of both serotonin and its metabolites have been associated with impulsive and aggressive behaviors in general⁹⁸⁻¹⁰² and in suicide¹⁰³⁻¹⁰⁶ and autism¹⁰⁷⁻¹¹¹ in particular, although these findings are still under debate¹¹².

Serotonin can be found in all human body tissues and fluids. In the CNS, it modulates a large number of cerebral, cognitive, hormonal and emotional functions. Mood, appetite and sleep, aggressive and sexual behaviors, locomotion, learning and memory consolidation are all regulated

by serotonergic neurons. Additionally, it regulates pulmonary and cardiovascular physiology, hemostasis, and endocrine functions. As a trophic factor, it is involved in neural development, homeostasis and plasticity. Serotonin is synthesized in dorsal raphe nuclei located in the primitive brainstem, but about 95% is found in blood platelets, insuring aggregation and vasoconstriction of blood vessels, and in enterochromaffin cells within the digestive apparatus¹¹³. The remaining synthesized neurotransmitters are delivered throughout the entire brain using outputs originating from the caudal raphe to innervate the spinal cord, while medial and dorsal raphe nuclei project toward almost all brain areas^{113,114} (for a map of the main serotonin projections in the brain and the spinal cord, see figure 7 in ref⁹⁶). Within the forebrain, the frontal cortex, together with the corpus striatum, the lateral region of the hypothalamus and the amygdala are innervated by projections issuing from the dorsal division of the nucleus centralis superior⁹⁶. An exhaustive listing of the areas innervated by the dorsal raphe is not relevant for this report, but the extent to which serotonin influences the overall brain activity by its ubiquitous presence demonstrates its pleiotropic role over all other monoamines¹¹⁵. All effects and regulatory processes controlled or modulated by serotonin necessitate its interaction with serotonin receptors.

Serotonin receptors

The serotonin receptors appear to be among the oldest neurotransmitter receptor system within the rhodopsin-like 7TMR subfamily^{11,116}. All subtypes are expressed in the CNS by nineteen genes (14 metabotropic receptors and 5 ionotropic receptor subunits) coding for seven different receptor types composed by nineteen different subtypes. Except for the 5-HT₃ receptor (constituted by 5 subunits 5-HT_{3(A,B,C,D,E)} forming a pentameric structure) which are a cys-loop ligand-gated ion channels activating inward K⁺/Na⁺ currents^{117,118}, all other receptors are metabotropic and classified in three categories according to their G protein signaling. The 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F} and the 5-HT_{5A}, 5-HT_{5B} receptors are inhibiting cAMP accumulation after release of the G $\alpha_{i/o}$ subunit following receptor activation. The 5-HT_{1C}R has been revealed to be the 5-HT_{2C}R

receptor. Conversely, the 5-HT₄, 5-HT₆ and 5-HT₇ are G_{α_s}-coupled and stimulate cAMP production in cells. The subtype 2 regroups the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} mostly coupled to production of IP3 and DAG after hydrolysis of PIP2 following activation of the G_{α_{q/11}} pathway, also stimulating PLC and PKC.

5-HT_{2A} receptor

The class A 5-HT_{2A} receptor, encoded by the *HTR2A* gene, is a subtype of the 5-hydroxytryptamine receptors type 2 and shares this subgroup with 5-HT_{2B} and 5-HT_{2C} receptors. These subgroup 2 receptors share 45-60% of sequence homology and are coupled to their canonical G_{α_{q/11}} signaling pathway¹¹⁹. Although found outside the CNS, a study provided evidence that serotonin-induced activation of 5-HT_{2A}R in cultured rat renal cells produced about 50% inhibition of forskolin-stimulated cAMP accumulation, a signal that was reversed by application of the antagonist ketanserin, indicating that this mainly G_{α_{q/11}} coupled receptor can activate G_{α_{i/o}} signaling pathway, at least in this peculiar primary culture¹²⁰.

The 5-HT_{2A}R is by far one of the most studied 7TMR and consequently, one of the most known serotonin receptor¹²¹. This is in part due to its atypical and unique pharmacology, as well as its wide distribution in the CNS and substantial involvement in neuropsychiatric disorders.

5-HT_{2A} receptor distribution

A recent study analyzed the human biodistribution of the 5-HT_{2A}R by positron emission tomography (PET) with two specific radioligands and showed a wide expression in numerous organs and tissues¹²². In the brain, it is well characterized and widely distributed in Rodents¹²³ and non-human Primates¹²⁴, and although mostly present in postsynaptic neurons¹²⁵⁻¹²⁷, its presynaptic activity has been demonstrated^{124,126-128}. It is also present on astrocytes^{127,129}. In rat and human, [³H]ketanserin radioligand binding co-distributes and overlaps with mRNA histochemistry demonstrating dense labelling of the major parts of the cerebral cortex regions, though denser in laminae I, IV and Va, the piriform and the entorhinal cortexes, as well as the

claustrum¹¹⁹. Using another 5-HT_{2A}R radioligand antagonist, [³H]MDL100,907, radiolabeling signals coupled to *in situ* hybridization (ISH) showed a matching overlap of both transcripts and protein in the rat brain, confirming most of the cortical regions previously described in Mammals and in Rodents, with a preference for the layer V of prefrontal cortex pyramidal neurons¹³⁰. Lesser densities are found in the basal ganglia, in particular the caudate-putamen nucleus (CPN) and the nucleus accumbens (NAc). The cerebellum seems exempt of signal across many studies and often used as a control or reference area^{119,131,132}, however some findings involved serotonin receptor localization and dysfunction in this area in humans and in rats^{133,134}. Caution must be taken about the specificity of the radioligands used to label expressed receptors prior to the development of [³H]MDL100,907^{114,135} and [¹¹C]-Cimbi-36¹³⁶, accounting for their technical limitations and caveats¹¹⁴.

In situ hybridization enabled to localize 5-HT_{2A}R mRNA in diverse cell types of the rat brain, mostly pyramidal neurons of the neocortex and the hippocampus, as well as interneurons of the piriform cortex¹³². The lamina IVc of the human striate cortex also showed positive signal. Immunoreactivity in the rat brain displays dense staining in the apical dendrites of pyramidal shaped neurons of the neocortex, as well as both parvalbumin positive and negative interneuron bodies¹³⁷.

The cellular and subcellular distribution of this receptor remains difficult to ascertain. Despite numerous examples of its localization both at the plasma membrane and within neuron somas, there is to date no definitive accordance on its spatial distribution either in heterologous cell systems or in native tissues. It is however accepted that 5-HT_{2A}R acts both intracellularly and as a prototypical plasma membranous 7TMR depending on the brain regions and/or the neuronal/peripheral function involved (see discussion below). Therefore, the localization of the 5-HT_{2A}R may affect both its trafficking and signaling properties, and vice-versa.

5-HT_{2A} receptor pharmacology

5-HT_{2A} receptor is the target of endogenous neuromodulator serotonin (5-HT). It is also targeted by atypical antipsychotics (AAP) such as clozapine, olanzapine, quetiapine, paliperidone, aripiprazole and risperidone, but also M100907, altanserin, mianserin, and ketanserin, acting as inverse agonists or neutral antagonists. It is also bound by agonists and partial agonists among a wide range of hallucinogenic compounds (HAL) which effects are psychedelic, phenethylamine-like, *e.g.* DOI, DOB, and indolamine-like, *e.g.* mescaline, dimethyltryptamine (DMT), psilocybin, lysergic acid diethylamide (LSD), acting as partial agonists^{138–140}. Remarkably, some compounds are not hallucinogenic (NHAL) (*e.g.* lisuride, ergotamine) despite high molecular similarity with HALs. 5-HT_{2A}R thus exhibits a singular pharmacology¹⁴¹, being the primary target of pro- and antipsychotic drugs^{114,140,142,143}. Such psychedelic compounds elicit response in humans that profoundly affects behavior and consciousness^{139,144}. In peak phases, this singular response is often compared to delusions and hallucinations that resemble acute positive symptoms of the schizophrenia spectrum disorder^{145,146}. However, while psilocybin provokes psychosis-like behavior in healthy volunteers via 5-HT_{2A}R activation¹³⁸, other agonists, including psilocybin, are currently considered as potential treatment for depression and other psychiatric conditions^{147–155}. This is indeed of particular interest for psychiatric disorders and related pharmacology, because HALs are employed to model psychosis in Rodents and in Humans^{138,156}. Activation of 5-HT_{2A}R is not sufficient to elicit hallucinogenic response, but it is necessary^{140,143,157}.

5-HT_{2A} receptor signaling and trafficking

Serotonin regulates essential functions in the CNS such as mood appetite and sleep, along with peripheral vasoconstriction and organs development. The 5-HT_{2A}R transduction model describes the G $\alpha_{q/11}$ -coupled signaling pathway upon ligand binding, stimulating PKC through PLC recruitment, to finally mobilize calcium release from the ER¹⁵⁸. The trafficking of the 5-HT_{2A}R has been described as atypical. Internalized by either agonists or antagonists, this is a specificity

hardly found in other classes and other types of 7TMR^{159,160}. The 5-HT_{2A}R is also thought to activate pathways differentially and promote agonist-directed trafficking of receptor stimulus¹⁶¹. Latter termed “biased agonism”, this functional selectivity has been shown to differentially signal downstream upon activation by either psychedelic or non-psychedelic agents. While canonical signaling through G $\alpha_{q/11}$ would be responsible for non-hallucinogenic-mediated downstream activation, hallucinogens require in addition to signal through pertussis toxin-sensitive G $\alpha_{i/o}$ pathways^{140,162}. Similar to this concept of signaling, it has also been shown that the 5-HT_{2A}R is at the center of a biased phosphorylation which provides additional trafficking and internalization selectivity through the β -arrestin dependent pathway¹⁶³. While ERK-dependent phosphorylation by HAL such as LSD and DOI is abolished by pretreatment with pertussis toxin, ergotamine and lisuride NHAL signaling remained unaffected, comforting the idea that two G protein-activated pathways are required to elicit hallucination-like behavior *in vivo*, using the Head-Twitch Response as a proxy of hallucinogenic intoxication response in mice¹⁶⁴) or the ERK signaling readout in pyramidal neurons, as well as in *in vitro* heterologous systems¹¹⁴.

5-HT_{2A} receptor oligomerization

The 5-HT_{2A}R has been shown to form homo/heterodimers^{165–170} and oligomers with other 7TMRs^{171–175}. Several lines of evidence have shown that 5-HT_{1A} and 5-HT_{2A} receptors, which are co-expressed in cortical pyramidal neurons, form an antagonistic receptor heteromer by modulating NMDAR currents¹¹⁴. 5-HT_{2A}R and D₂R have been shown to associate as a functional receptor heteromer eliciting a signaling crosstalk signaling in cells^{172,176}.

Physiopathology of the 5-HT_{2A} receptor

5-HT_{2A} receptor is involved in the pathophysiology of schizophrenia-like phenotypes, depression and suicide, to name a few^{131,177–187}. It has been linked along with other subtypes to physiopathology of psychiatric and cognitive disorders, such as mood disorders and depression, suicide and schizophrenia, associative learning, where preferentially affecting areas regrouped in

the neocortex, in which the prefrontal cortex (PFC) seems to play an important role^{124,125,143,183,187,188}. The list of disorders implicating the 5-HT_{2A}R is long, and it is clear that the receptor regulate many diverse systems and functions in the body and the brain; it comprises Alzheimer disease, autism, Parkinson disease, obsessive compulsive disorder, addiction, suicide, cardiovascular lung and skin disorders, as well as diabetes and inflammation, to name a few. What is unclear though, is how dysregulated is the receptor in many neuropsychiatric disorders. Discrepancies about the level of 5-HT_{2A} protein and mRNA, often in post-mortem human brains, is still a matter of debate^{185,189}.

Glutamate

Neurotransmission in the mammalian brain is usually depicted as a synchrony between excitatory and inhibitory electrochemical signals. L-glutamate is known to be the main excitatory^{190,191} and the most abundant¹⁹² non-essential amino acid in the mammalian CNS, while GABA relays inhibitory neurotransmission. Glutamate concentration is high in the CNS, partly because it is not solely used for excitatory neurotransmission, thus holding other metabolic activities in glia and neurons¹⁹³. Both glutamate and GABA are synthesized from glutamine precursor. Glutamine is synthesized from glutamate by ATP-dependent glutamine synthetase in astrocytes. Glutamine is exported to the neuron by glycine/glutamine transporters. In the neuron, it is then converted into glutamate by the phosphate-activated glutaminase¹⁹⁴. Glutamate is decarboxylated by the glutamic acid decarboxylase in presence of cofactor pyridoxal phosphate and converted into GABA. After vesicular glutamate transporter 1 (VGLUT-1)-mediated packaging of glutamate in synaptic vesicles, it is released to bind both post synaptic ionotropic and post/presynaptic metabotropic glutamate receptors/autoreceptors respectively. This results in modulation of Ca²⁺ release, acting as a second messenger required for the activation of signaling cascades. It is removed from the synaptic cleft back to the astrocytes through transmembranous excitatory amino acid transporter (EAAT), thus completing the glutamate-GABA/glutamine cycle. It also suggests

the existence of different pools of glutamate-containing neural cells, providing the resources to tightly regulate glutamine, glutamate and GABA homeostasis in the brain. Glutamate concentration is maximal intracellularly, and extracellular increase of glutamate produce neuron's excitotoxicity¹⁹⁵⁻¹⁹⁷.

Glutamate receptors

As mentioned above, there are two types of glutamate receptors expressed in the CNS. Ionotropic glutamate receptors (iGluRs) are fast-acting ligand-gated ion channels that are non-selective cations channels, thus mainly exchanging Na⁺ and K⁺, together with lesser concentration of Ca²⁺ (in the case of NMDAR). They are termed according to their main activating ligand: NMDA (*N*-methyl-D-aspartate), AMPA (α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), and kainate (kainic acid) receptors. As cation channel, they carry out excitatory post synaptic currents resulting in cell depolarization and in optimal cases, creation of an action potential. As their related metabotropic cousins, iGluRs can be both pre- and post-synaptic, complexifying the simplistic black and white view on presynaptic mGluRs and postsynaptic iGluRs¹⁹⁸. Slow modulation of glutamate release is accomplished by metabotropic glutamate receptors (mGluRs). Indeed, the role of mGluRs differs from that of fast-acting ion channels, because they can be sensitive to both orthosteric and allosteric pharmacological compounds and drugs, thus changing the excitability of the postsynaptic neurons. Additionally, their G protein coupling triggers intracellular messengers that affect neuron homeostasis, function and transmission.

To date, the class C 7TMRs mGluRs are categorized into 3 different groups according to their G protein coupling and sequence homology^{199,200}. Splicing variants are common in this subfamily (subscript letters). Group I contains G α_q/α_s -mediated signaling subtypes, mGlu1_(a, b, c, d, e, f), taste-mGluR1 and mGlu5_(a, b) receptors. Group II and III are mainly inhibitory-mediated signaling, coupled to G α_i protein, decreasing adenylyl cyclase activity, stimulating K⁺ inward ion channels and altering Ca²⁺ currents, thus negatively affecting neurons firing and excitability by an overall

reduction of glutamate neurotransmission. Group III is composed by mGlu4 and taste-mGlu4 (a shortened, less sensitive version of mGluR4²⁰¹) receptors, the retinal mGlu6_(a, b, c) and the presynaptic mGlu7_(a, b, c, d, e) and mGlu8_(a, b, c) receptors. mGlu2 and mGlu3_(A2, A4, A2A3) receptors form the Group II, sharing homology of structure and Gα_q protein signaling and pharmacological characteristics. They all bear a large extracellular domain responsible for the binding of orthosteric ligands, the VFT.

mGlu2, mGlu3 receptors distribution

Group II mGluRs are encoded by the *GRM2* and the *GRM3* genes. The homology of sequence between the two genes approximates 67%, with 70% among the conserved regions between mGluR1, 2, 3, and 4, especially at the c-terminus and at the VFT domain. Besides, they do not share any similarity with the other 7TMR classes²⁰². *In situ* hybridization studies indicate a wide distribution of mGluR2 in discrete cell types in the rat CNS. It is high in the olfactory bulb and the cerebral cortex. Immunohistochemistry studies with a specific mGluR2/3 antibody showed expression of both receptors in many regions of the mouse brain with variable expression patterns and intensities. This suggests that a wide array of subregions where both receptors are coexpressed and possibly interact²⁰³. Additionally, the cell types are distinguishable among regions but both pre- and post-synaptic neurons are positive for either mRNAs or protein expression of mGluR2/3, as well as substantial glial expression²⁰³. In the cerebral cortex, layer I to IV were highly stained, as well as deeper regions in the layer V and VI of medium and pyramidal neurons²⁰³. Other important reactive regions were the piriform, frontal and entorhinal cortices, subregions of the hippocampus, the thalamus, and main nuclei of the basal ganglia such as nucleus accumbens and caudate putamen. A study using autoradiography coupled to electrophysiology with the selective mGluR2/3 radioagonist [³H]LY459477 in WT, mGluR2^{-/-} and mGluR3^{-/-} mice, to specifically address distribution of each receptor, matched most of the regions cited above in CD-1 mice²⁰⁴. In the basal ganglia, no signal could be found outside the substantia

nigra, though both caudate-putamen and nucleus accumbens shell were mGluR2 positive, in accordance with monoclonal antibody immunoreactivity in both mouse and rat, which also detected mGluR2 in the ventral tegmental area (VTA)²⁰⁵. mGluR3 was also found in the same subcortical regions with high immunolabeling ultrastructure resolution²⁰⁶. The claustrum was also positive for mGluR2 and mGluR3 radioactivity with different amounts, although the above study using immunohistochemistry technique reported a weak signal²⁰⁵. This study²⁰⁴ was also able to differentiate regions expressing either mGluR2, such as prefrontal cortex, and mGluR3, such as dorsolateral and entorhinal cortexes.

Whereas electron microscopy study found mGluR2/3 staining in many glial processes, vesicles of putative excitatory synapses, presynaptic terminals near synaptic clefts of the layer I cerebral cortex, signal was also high in postsynaptic small spine synapses. Glial staining was detected in all layers, although the authors commented on the difficulty to delineate accurately the processes that seemed positive²⁰³. Conversely, no glial cell expressing mGluR2 were found with monoclonal immunostaining technique at the EM resolution²⁰⁵. Subsequently, other EM studies demonstrated the presence of mGluR2/3 in astrocytic bodies that ensheathed glutamatergic-like synapses, and confirmed the localization of both receptors indistinctly in pre- and post-synaptic processes such as axons, dendrites and spines of the dorsolateral prefrontal cortex of non-human Primates²⁰⁷. In the same region of the same animal model, it was specified the relative amount and presence of both mGluR2 and mGluR3. Because this region is critical for many neurodegenerative and psychiatric disorders, the localization of group II mGluRs invests a substantial importance. mGluR2 is mostly reported as a presynaptic terminals autoreceptor^{200,208–210}, and, to a lesser extent, on a variety of spines, but mostly as an extrasynaptic receptor. It was also clearly present in astrocytes although previously thought to be absent in glia²⁰⁵; mGluR3 seemed to be the prominent glial receptor²¹¹. mGluR3 was mostly present on astrocytes and postsynaptic spines, where it was shown to negatively modulate cAMP production²¹². These findings suggested, in the

context of delayed neurons firing in the layer II of the dorsolateral prefrontal cortex, that mGluR2 and mGluR3 have distinct functional roles in distinct regions of the tripartite synapse, although it was previously assumed that they could share similar brain functions, where mGluR2 was assumed to reduce neurons sensitivity together with other $G\alpha_i$ -coupled mGluRs^{200,213,214}, while mGluR3 would act as a potent mechanism of reuptake glutamate following vesicular release in the extracellular field of excitatory glutamatergic terminals¹⁹⁷.

Most of these regions also express the 5-HT_{2A}R, in particular the layer Va of the prefrontal cortex that showed dual labelling of [³H]LY459477 and either [¹²⁵I]DOI or [³H]M100907 (in mGluR3 KO mice)²⁰⁴, indicating that 5-HT_{2A}R and mGluR2 co-distributed in critical regions involved in neuropsychiatric disorders, as previously reported¹³⁰. Both receptors are expressed and detected in thalamic regions, the prefrontal cortex, hippocampal subregions such as the entorhinal cortex, as well as the dorsal and ventral striatum and the amygdala²⁰⁴.

mGlu2, mGlu3 receptors pharmacology

Group II mGluRs can be sensitive to the same agonists as of their ionotropic cousins. Glutamate is obviously one of them, but also quisqualate, which initially served as a selective agonist for mGluR2 and mGluR3 with differential cellular electrophysiology in cloned expressed receptors^{200,202}. As of today, and to our knowledge, agonists sufficiently selective to distinguish mGluR2 from mGluR3 are still not available. This is due to the sequence proximity at the extracellular orthosteric binding site. Pharmacological design has however progressed in the selectivity of this Group II agonists, and a compound, partial agonist at mGluR2 and antagonist at mGluR3 has been developed and tested²¹⁵. Another compound, N-acetylaspartylglutamate (NAAG), the most abundant peptide in the mammalian brain, elicited high mGluR3 selectivity but not at other mGluRs, including mGluR2, implicated in synaptic plasticity and neuroprotection against excitotoxicity^{216,217}. Although selective, it remains an endogenous mGlu3 receptor agonist. Other agents could possibly be used in some cases, but the constraints over group II mGluRs

pharmacology limit studies that would be able to distinguish the exact role held by each receptor. This issue can be somewhat overcome by the recent availability of positive and negative allosteric modulators, (PAM or ago-PAM and NAM respectively^{218–220}).

The development of highly selective orthosteric mGluR2/3 agonists such as LY379268, LY354740, LY2140023 (the LY404039 prodrug) and LY404039 enabled to refine the function of each receptor in experiments *in vitro*, but also in clinical trials to treat neuropsychiatric disorders such as schizophrenia and anxiety, stress and neurodegeneration, and even cancer and pain management, to name a few^{221–235}. Additionally, the affinity of such drugs can differ from one brain area to another, or across different neuronal cell types²³⁶. LY379268, LY2140023 (the LY404039 prodrug) and LY404039 suppress the behavioral and physiological effects of psychotomimetic drugs and serotonergic hallucinogenic drugs, both enhancers of glutamate release, and show their efficacy in the treatment of schizophrenia²¹¹. The metabotropic glutamate receptors, including mGluR2 and mGluR3 are considered new targets for their antipsychotic activity and, despite undergone a hold in clinical application, their effects on the positive, negative and cognitive symptoms of schizophrenia continue to raise hopes^{170,218,226,234,237–240}.

mGlu2, mGlu3 receptors signaling

Because of their similarity in residues sequence, cognate G protein and pharmacology, it has long been assumed that they also share function and similar roles in the brain, despite being localized in different amounts in different localizations and cellular types. The overall decrease of glutamatergic excitability follows the reduction of presynaptic vesicle release caused by the inhibition of Ca²⁺ inward currents. Moreover, ligand-induced dissociation of the G α_i protein $\beta\gamma$ subunit can signal through a wide array of second messengers, typically the mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated protein kinases (ERK) pathways, mediated by the phosphatidylinositol 3 kinase (PI3K)²⁴¹. This is a fundamental signaling route involved in critical cellular responses such as cell proliferation and transcription factors regulation.

The loss of function remains a pertinent strategy to decipher to study the role of a protein or a gene. As mGluR2 and mGluR3 being very close on many aspects, the use of knock-out animal models, or even double knock-outs, has provided important information about the precise functions of each receptor in the rodent brain. It offers a unique way to study such features, coping with the issue of non-selective agonists within the mGluRs group II. To that end, several studies have attempted to discern the specific role of mGluR2 over mGluR3 in pain²⁴², cognition, arousal and anxiety^{239,243}, and the circadian system²⁴⁴. With regards to circadian rhythm and sleep disruption, it is a comorbid symptom of schizophrenia, affecting 30-80% of patients²⁴⁵. When mice were depleted of both mGluR2 and mGluR3, their sleep was perturbed, reduced and fragmented, implicating group II mGluRs in sleep regulation²⁴⁴. It also suggests a possible link between the implication of both receptors in schizophrenia and such perturbations of fundamental physiology²⁴⁶. The above-mentioned mGluR2 agonists are used in clinical trial to test a new kind of antipsychotics or anxiolytic medications, among other targeted neuropsychiatric disorders. They were suggested to show reduced heavy side effects associated with atypical antipsychotics or benzodiazepines. Although there is to date no correlation between the *GRM2* gene and schizophrenia, *GRM3* abnormalities however have been detected multiple times in genome of patients suffering from this syndrome.

mGlu2 receptor oligomerization

As mentioned above, class C 7TMRs are obligate dimers though their function in signaling G protein is mitigate by their stoichiometry. They also form heterodimer within their class and across subtypes, including mGluR2/mGluR3 heterodimers^{240,247-249}.

Pathophysiology of the mGlu2 receptor

Some authors have associated schizophrenic phenotype with mutations within mGlu receptor genes *GRM2* and *GRM3*²¹¹. Altered dimerization of type 3 has been implicated in schizophrenia²⁵⁰. Structural and functional interactions have been reported for the mGluR2-5-HT_{2A}

heterocomplex^{183,251–254}. Both receptors, but especially mGluR2 has been the center of intense researches for its potential antipsychotic activity with potentially less side effects.

Summary

Given the number of matched regions expressing either 5-HT_{2A}R, mGluR2 and mGluR3, there is a high probability that a single neuron expresses these receptors or receives input from one and expresses the second. Because their synaptic localization is also pre- and postsynaptic, as well as glial, (*i.e.* all membranes and soma of the tripartite synapse), it increases the probability for a functional role of the interaction formed by 5-HT_{2A}R and mGluR2 or by mGluR2 and mGluR3 (or by 5-HT_{2A}R and mGluR3 even if they do not seem to interact directly¹⁸³). Together this information provides evidence and leads in the involvement of 5-HT_{2A} and mGlu2 receptors, in participating in brain functions and their alterations. They co-distribute and colocalize, their antagonistic signaling is physiologically relevant and different from each other canonical signaling^{255–257}. Moreover, the absence of one affects the behavioral effect of the other. All those criteria are consistent to the required properties necessitated by a receptor heteromer⁸⁶.

5-HT_{2A}-mGlu2 receptor heterocomplex

Those two receptors have been reported to play independently and then likely as a heterocomplex, significant roles in psychiatric disorders and especially schizophrenia¹⁸³. As previously mentioned, the PFC represents the area of interest demonstrated by previous electrophysiological studies that suggested an antagonistic crosstalk¹²⁵. *In situ* hybridization experiments reported that both receptors are co-transcribed in pyramidal neurons of mouse cortex and human PFC, where they co-immunoprecipitate and show close molecular and functional interactions revealed by B/FRET analyses coupled to functional assays, excluding 5-HT_{2A}/mGluR3 assembling^{183,252}. Electron microscopy by immunogold particles colocalization confirmed close proximity in ultrastructural resolution of pre- and post-synaptic elements²⁵². Additionally, this interclass association modulates Gα_q and Gα_i pathways in an antagonistic and specific manner and support evidences

of heteromeric structures with physiological relevance^{67,69,70,251,254}. The structure interface responsible for their heteromerization has been established by a mutation study of the TM4 of the glutamate receptor²⁵². In follow up experiments, these findings have been corroborated using photocrosslinking assay, where the covalent linkage between the unnatural amino acids in the TM regions occurs within a distance of 2-4 Å⁸⁹.

7TMR trafficking and biosynthesis

7TMRs are synthesized throughout the maturation pathway from ribosomal translation processes at the edge of the endoplasmic reticulum where they are folded and glycosylated, undergoing quality control checkpoints²⁵⁸. The Golgi apparatus constitutes the exit pathway as an active post-translational element, finalizing 7TMR biosynthesis. This export process is highly regulated by cargo and scaffold proteins^{70,258}. 7TMRs are described as actual signaling units when reaching the plasma membrane, but increasing evidence suggest signaling can occur from endosomes²⁵⁹. It is also of particular interest that 7TMR signaling may occur during their trafficking stages²⁶⁰. Although it is not the case for all of them, as β -arrestin-independent mechanisms have been described^{158,261–263}, most 7TMRs undergo ligand-induced endocytosis through β -arrestin/dynamin/clathrin-coated-pits interactions, a 7TMR core process. Among 7TMR subcellular trafficking regulators, the Ras-family small-GTPases Rab proteins are ubiquitous^{258,263}. In particular, Rab5 regulates endocytic/early endosomal processes, Rab4 and Rab11 control rapid and slow recycling from recycling endosomes, Rab7 is a regulator of the late endosomes and the lysosomal degradation pathway taken by ubiquitylated proteins. Localized throughout specific intracellular stages, they can therefore serve as exploratory molecules to characterize GPCRs trafficking pathways.

Rab proteins

Navigating through the ultra-dense network of actin fibers and microtubules necessitates the guidance of proteins and cargoes to their appropriate destinations. These two last words suggest

specific recognition and trajectories taken by the cargoes. This is accomplished by a set of proteins counting around 60 subtypes: Rab proteins are small monomeric G proteins constituting the largest group of the Ras-like protein superfamily and hold a common GTPase activity. They orchestrate trafficking events between donor and target membranes. Each Rab protein is a resident of the organelle membrane they regulate, providing an identity to the subcellular compartment²⁶⁴. Organelles are also defined by specific scaffold proteins that anchor them to the cytoskeleton, but also by the lipid composition of their membrane layers. Rab1 interacts with the Golgi apparatus and the ER protein complexes each dedicated to their organelles functioning, to activate the enviroing SNAREs, and to “read” compartments scaffolds to facilitate transport in between the ER and the Golgi apparatus²⁶⁴.

The duration the GTPase activation cycle is regulated by guanine nucleotide exchange factor (GEFs), who sustains the GDP/GTP exchange, and terminated by hydrolysis of GTP carried out by GTPase activating proteins (GAPs). Rab proteins partner with Arf family to interact with the cell cytoskeleton and in particular with the motor proteins that mediate the transportation of cargoes along the microtubule/actin networks, in both directions^{265,266}. For instance, Rab7a promotes the trafficking of cargoes by recruiting the dynein/dynactin motor protein complex to the late endosomes, to direct the enriched vesicles toward the perinuclear areas²⁶⁷. Similarly, Rab5 binds to the early endosome antigen 1 protein (EEA1) to recruit the early endosome target, with concomitant association to PI3P. Rab5 also recruit membrane effectors that allow interaction with SNAREs for further engage membrane fusion^{268,269}.

Rab proteins, besides providing identity and mobility to their respective organelles, can interact with specific GEFs and GAPs of the next Rab protein, thus creating “Rab cascades” that will orient and organize transitions between compartments. In particular, it has been proposed that Rab7 is present on early Rab5 endosomes. Rab7 buds then grows on the Rab5 early endosome membrane and likely interacts with converter proteins. These proteins would turn off the recycling

properties of the sorting early endosomes (which are defined by Rab4 and Rab11), and convert it to a late endosome²⁷⁰. Rab5 is displaced and Rab7 is activated. Because effector protein GEF can recognize an increase in Rab5 and PIP3, this gives selectivity to the process. It is also coincident with the membrane recruitment, therefore allowing Rab5 displacement toward Rab7 at a specific time in a specific location²⁷¹. This example demonstrates a mechanism that synchronizes protein trafficking in time and space.

Considered as master regulators²⁷², Rab proteins regulate vesicle sorting, docking, budding, tethering and membrane fusion. They define the secretory and the endocytic routes by interacting with specific components along these pathways. They also specify the microdomain occupied by the organelle they control, underneath the plasma membrane. Rab proteins also regulate the trafficking of 7TMRs, and it has been demonstrated that the basic four Rab proteins (4, 5, 7 and 11) direct and orient internalization and recycling of several 7TMRs²⁷³. Following β -arrestin-dependent desensitization after being internalized, 7TMRs follow multiple routes in accordance with their interacting Rab protein, effector, or β -arrestin type. Indeed, it has been shown that 7TMRs can follow two different arrestin-mediated pathways²⁷⁴. Following ligand-induced internalization, β_2 AR has been shown to traffic through Rab5 endosomes²⁷⁵. Overstimulation of Rab5 by expressing a dominant positive mutant affect β_2 AR trafficking through early endosomes. Conversely, dominant negative Rab5 impairs early trafficking of the receptor. This was also the case for the D₂R, which is regulated by dynamin following phosphorylation by GRK2²⁷⁶. Interestingly, the class A angiotensin receptor 1A (AT_{1A}R) has been shown to use both β -arrestin-dependent and -independent pathways without being regulated by Rab5, although it is bound by the receptor. This suggests that Rab5 mediates different endocytic mechanisms²⁷⁷.

Endosomes

Endosomes are dynamic cytoplasmic vesicles acting as trafficking regulators and sorting platform. Their importance has generated much attention as targets for novel therapeutics^{278,279}. They

provide the necessary space and means to regulate the trafficking, and identify not only the nature of the entrant and recycled particles, endocytosed receptors and lipids, but also a platform for receptor signaling, whether they come from the plasma membrane or from the secretory pathway. They appear poorly studied despite their critical role in protein trafficking, as well as in receptor signaling²⁸⁰.

Usually categorized as early (EE), sorting (SE), recycling (RE), or late endosomes (LE), they adopt different shapes structured around the cytoskeleton with specific cellular functions. They can be ramified to tubular extensions (EE, multi-vesicular body MVB) or simply remain spherical-like, mostly depending on their function and associated motility, sustained over the microtubular network²⁸¹. The pH gradient between the cytoplasm and the lumen of intracellular vesicles provides the differential ionic concentration that serves to dissociate the ligand from its receptor, the former often directed to degradation whereas the latter can either be rerouted to the plasma membrane or follows the same fate as its ligand²⁸². The role of each endosome also depends on their localization underneath the plasma membrane, constituting functional microdomains. Their identity is determined by the combination of regulatory proteins and lipids that are unique to each specialized endosome²⁸³.

A pH gradient spans through the cytoplasm from the plasma membrane to deeper compartments. The higher pH is found right under the plasma membrane, where early endosomes control the endocytic trafficking of newly endocytosed elements²⁸⁴ (recycling endosomes have a slightly higher pH). They are thought to constitute the first and only entry point of endocytosed proteins/lipids independently of the internalization mode, such as clathrin-mediated and lipid raft-mediated endocytosis or pinocytosis, as well as the first recycling platform^{283,285}. To that end, their structure consists in several cisternal areas associated with budding vesicles and thin tubular emanations (for a stunning visualization by electron microscopy of the EE structure see ref²⁸³). A complex association and interplay of specific resident proteins participate to endosomal transport,

morphology, sequestration, docking, etc.²⁸³. Among them, Rab5 and its effectors appears to be the main regulators that take on several functions such as spatial organization, docking affectation from either trans-Golgi or plasma membrane. Membrane budding, fission and interaction with the cytoskeletal components are also among Rab5 attributes (see above)^{268,286,287}.

The model describing the compartmentalization of endosomes has been proposed by early studies that investigated fluorescent markers routing and chased their cellular destination and trafficking in a timely manner, usually depicting a common network of specialized vesicles as a general trafficking model²⁸⁸. Although it was assumed that integral proteins, ion channels and receptors underwent at least two types of internalization processes, degradation or recycling, the putative motion of different membrane-based vesicles was described as a bulk flow process, without specificity²⁸⁸. This model has been refined to the point that there is no longer such a bulk flow of membranes that fusion and interact, but rather a complex interplay orchestrated by specialized proteins and adaptors along endosomal-mediated tubular networks²⁸⁹, sorting motifs attached to internalized cargoes that orient their route according to the cell needs²⁹⁰.

EE provide both fast and slow recycling pathways²⁸⁵, though evidence suggested that LE can also recycle cargoes. Lumen pH is lower in LE, which link endocytosed cargoes to degradation pathway through EE sorting^{284,291}. Within the EE or the LE, cargoes routed to the recycling pathway should avoid inclusion in intermediate degradative vesicles (intraluminal vesicles) to be able to reach back to the plasma membrane, either by the slow or the fast recycling routes²⁹⁰. Thus, LE, also identified to MVB, function as sorting dispatchers²⁹⁰.

A major shift in envisioning receptor signaling was provided by findings in regard to intracellular signaling, and more precisely, on signaling from endosomes. Receptor endocytosis has been studied as a termination mechanism to regulate available receptor density at the cell surface and mitigate cell responsiveness following agonist stimulation. Receptor desensitization and resensitization are not general mechanisms that all 7TMRs undergo. Although β -arrestin-

dependent internalization of clathrin-coated pits and adapter protein-2 complex (AP-2), in response to GRK-mediated receptor phosphorylation, plays a major role in 7TMR internalization, besides being the most well characterized system, some receptor types are recycled, some are not and get degraded²⁹². Recycling endosomes employs several Rab proteins (Rab5, 4, and 11) while late endosomes are often related to degradation pathways and mostly regulated by Rab7 (and Rab9). The early or sorting endosome is the first encountered container of most internalized proteins and lipids²⁸⁵.

Together, this information suggests that the endosomal network is finely regulated by resident and adaptor proteins, and that, together with the architecture of the specified endosomes, produces subcellular microdomains composed by the vesicles tubular extensions over the actin/microtubule skeleton²⁹³. Each microdomain, regulated by a specific Rab protein, serves as orientation platforms to either degrade or recycle cargoes that are either endocytosed or synthesized.

Schizophrenia

Schizophrenia, or schizophrenia spectrum disorder (or syndrome), is a serious multifactorial mental illness affecting up to 0.65% of the USA population and 20 million people worldwide²⁹⁴ (up to 0.75%), with chronic, and in great majority, life-lasting social, emotional and cognitive alterations. Clinical symptoms are often grouped as so-called negative, positive, and cognitive^{295,296}. Studies and definitions converge to acknowledge that a combination of genetic susceptibilities and harmful environmental factors participate to its etiology, which remains unknown. Nonetheless, despite a raise in the prevalence of brain disorders and consequent increase economic and social burden²⁹⁷⁻³⁰⁰, where schizophrenia costs are disproportionately high³⁰¹, pharmacological companies have halted avenues of research for claimed financial reasons³⁰²⁻³⁰⁸, creating a gap between a steady clinical and fundamental research and the availability of commercialized medications.

The classical hypothesis of dopaminergic hyperactivity gained attention since the antipsychotic activity of several commercialized dopamine receptor (DR) antagonists (typical) would correct some of the positive symptoms, especially delusions and hallucinations. The dopamine hypothesis suggests that there is an imbalance of the dopaminergic tone in the prefrontal cortex that can be rectified by treatment with D₂R antagonists, such as chlorpromazine, pioneer molecule to treat positive symptoms³⁰⁹. Compared to the first generation antipsychotics (the typical D₂R/D₃R antagonists), atypical antipsychotics (AAP) have more affinity for the 5-HT_{2A}R. Similarly, such pharmacological evidence fashioned the serotonin hypothesis, also based on the resemblance between serotonin receptor agonists that produce psychosis-like effects on humans. These molecules exhibit substantial off target occupancy and are considered as “*dirty drugs with rich pharmacology*”³¹⁰, carrying at the same time, important side effects. They are yet prescribed as first line compounds to treat schizophrenia’s positive symptoms, with moderate improvements of negative and cognitive ones. A third of subjects do not respond to AAP^{311,312}. Although the classical theories on the onset and the etiology of the disorder remain up to date, the scope has yet evolved toward other signaling pathways such as glutamate and GABA; the implication of a combination of neurotransmitter systems rather than a single defective gene or pathway could shed light on the disorder origin and mechanisms^{309,313,314}. Indeed, recent findings propose that a heterogeneous genetic network causes distinct clinical syndromes³¹⁵, as conveyed by years of research on the implication of several genes that regulate and express 7TMRs, receptor trafficking and neurotransmission, synaptic and structural plasticity^{316–320}.

The variability of symptoms combined to the diversity of phenotypes resulting from clinical and genetic observations conflict with a single disease paradigm, thus single medication are unlikely to treat the full spectrum that characterize the disorder³²¹. The absence of a uniform syndrome renders difficult the delivery of accurate, specific and reproducible diagnostics^{322–325}, which also rely on definitions and clinical practices³²⁶. For instance, some criteria can overlap with those of

bipolar disorder³²⁷ that may affect drug indication and assistance to patients^{328,329}, who eventually cope with long term/life lasting treatments, if they find access to them^{295,296}.

It is nowadays a common concern as to challenge the single disease paradigm^{297,330}, *i.e.* embrace several theories about the disorder to provide novel and more efficient medications. This was the case for atypical antipsychotics, who have affinity for both 5-HT_{2A}R and D₂R, and together increase the beneficial effects on the disorder process and evolution, rather than just on symptoms^{311,331–333}. The glutamatergic system has also gained in importance with the findings related to altered NMDAR-mediated glutamatergic neurotransmission, signaling and trafficking. NMDAR dysfunction on GABAergic neurons implicate a hyperglutamatergic stimulation responsible for cognitive impairments and other deleterious effects found in schizophrenia. Agonizing the mGluR/2/3 pathway potentially rectifies the glutamatergic overstimulation by reducing the presynaptic release of glutamate on excitatory neurons, as a compensatory mechanism²³³. Those agonists have been found to relieve all 3 symptoms type in patient with schizophrenia³³⁴. It was believed that such molecules held a novel and promising mechanism of action that could be beneficial to patients^{226,233}.

Hypothesis

Investigations in post-mortem schizophrenic prefrontal cortexes suggested that 5-HT_{2A}R specific binding is increased yet rectified in treated subjects with AAPs. However, mGluR2 remained downregulated in both untreated and treated schizophrenic subjects. We thus hypothesize that biosynthesis and endocytosis of interacting 5-HT_{2A}R and mGluR2 may play a role in their common and linked dysregulation. Trafficking and signaling being entangled, endocytosis and biosynthesis of 7TMR complexes control their cellular localization in time and space. Conversely, trafficking failure implies signaling impairments, which eventually result in a host of pathologies. The objective of the following study is to elucidate in *in vitro* and *ex vivo* models, the maturation and

the ligand-dependent trafficking mechanisms regulating the 5-HT_{2A}R-mGluR2 signaling complex to further explicit the effect of their imbalance in schizophrenia.

Aims

Aim 1: 5-HT_{2A}R modulates mGluR2 post-endocytic sorting. Hypothesis: Pharmacological modulation of 5-HT_{2A}R affects mGluR2 sorting and function through Rab-mediated trafficking pathways.

Aim 2: Effects of 5-HT_{2A}R antagonists/inverse agonists (antipsychotic) and hallucinogenic agonists (propsychotic) on 5-HT_{2A}R-mediated trafficking of mGluR2. Hypotheses: 5-HT_{2A}R and mGluR2 heterocomplex is affected by clozapine, as well as mGluR2 trafficking. Psychedelic agents promote Rab5-dependent pathway of mGluR2, while non-psychedelic agents do not.

Aim 3: 5-HT_{2A}R and mGluR2 are assembled as a GPCR complex in the biosynthetic pathway. Hypothesis: 5-HT_{2A}R and mGluR2 structurally associate in the biosynthetic pathway. BiFC of 5-HT_{2A}R and mGluR2 would be detected in both the endoplasmic reticulum and the Golgi apparatus.

Materials and Methods

Drugs

(±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI), 5-hydroxytryptamine hydrochloride (5-HT; serotonin), Lysergic acid diethylamide (LSD), Ergotamine, GDP, and GTPγS were purchased from Sigma-Aldrich. (1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), clozapine, (*R*)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol (M100907; MDL 100,907; volinanserin), 3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone hydrochloride (altanserin), L-glutamic acid, 3-hydroxynaphthalene-2-carboxylic acid (3,4-dihydroxybenzylidene)hydrazide (dynasore), [8β(*S*)]-9,10-didehydro-*N*-[1-(hydroxymethyl)propyl]-1,6-dimethylergoline-8-carboxamide maleate (methysergide) and Lisuride were purchased from Tocris Bioscience. [³H]Ketanserin and [³⁵S]GTPγS were obtained from PerkinElmer Life and Analytical Sciences. [³H]LY341495 was purchased from American Radiolabeled Chemicals. All other chemicals were obtained from standard vendors.

Plasmid Construction

All PCR assays were performed with PfuUltra Hotstart Polymerase (Stratagene) in a Mastercycler Ep Gradient Auto thermal cycler (Eppendorf). Cycling conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min/kb of amplicon, with an initial denaturation/activation step at 94°C for 2 min and a final extension step of 72°C for 7 min. All the constructs were confirmed by DNA sequencing. The following constructs have been previously described^{183,251,252}: human 5-HT_{2A}R N-terminally tagged with the c-Myc epitope (pcDNA3.1-c-Myc-5-HT_{2A}R), human 5-HT_{2A}R N-terminally tagged with the c-Myc epitope and C-terminally tagged with enhanced cyan fluorescent protein (pcDNA3.1-c-Myc-5-HT_{2A}R-eCFP), human 5-HT_{2A}R N-terminally tagged with the c-Myc epitope and C-terminally tagged with mCitrine (pcDNA3.1-c-Myc-5-HT_{2A}R-mCitrine), human 5-HT_{2A}R N-terminally tagged with the c-Myc epitope and C-terminally tagged with mCherry

(pcDNA3.1-c-Myc-5-HT_{2A}R-mCherry), human 5-HT_{2A}R N-terminally tagged with the c-Myc epitope and C-terminally tagged with eYFP (pcDNA3.1-c-Myc 5-HT_{2A}R-eYFP), human mGluR2 C-terminally tagged with eYFP (pcDNA3.1-mGluR2-eYFP), human mGluR2 C-terminally tagged with eCFP (pcDNA3.1-mGluR2-eCFP), human mGluR3 C-terminally tagged with eYFP (pcDNA3.1-mGluR3-eYFP), human mGluR2 with substitution of residues A677^{4.40}, A681^{4.44} and A685^{4.48} in mGluR2 for S686^{4.40}, F690^{4.44} and G694^{4.48} in mGluR3 C-terminally tagged with eYFP (pcDNA3.1-mGluR2 Δ TM4; formerly named pcDNA3.1-mGluR2 Δ TM4N). The construct 5-HT_{2A}R-eCFP was subcloned into the vector pcDNA5/FRT/TO (Invitrogen) for the subsequent generation of Flp-In T-REx HEK293 cells, as we have previously reported³³⁵. For BiFC studies, constructs were generated by subcloning the sequence encoding the N-terminal 172-amino acid fragment (mCi-N172) or the C-terminal 67 amino acid fragment of mCitrine (mCi-C76), as we have previously described [see ⁴⁰]. The constructs pcDNA3.1-mCi-N172, pcDNA3.1-mCi-C67, pcDNA3.1-mGluR2-mCi-N172 and pcDNA3.1-mGluR2-mCi-C67 have been previously described²⁵¹. The form of 5-HT_{2A}R C-terminally tagged with eCFP was digested with NheI and BamHI and subcloned into the same restriction sites of pcDNA3.1-mCi-N172 and pcDNA3.1-mCi-C67 to generate pcDNA3.1-5-HT_{2A}R-mCi-N172 and pcDNA3.1-5-HT_{2A}R-mCi-C67. The chimeric G α protein G α _{qi9} has been previously reported³³⁶. FRET calibration was performed on parental HEK293 cells transfected with either pcDNA3.1-eCFP, pcDNA3.1-eYFP, or the tandem plasmid pcDNA3.1-eCFP-eYFP.

Transient Transfection of HEK293 cells

Human embryonic kidney (HEK293) cells (ATCC: CRL-1573) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) dialyzed fetal bovine serum (dFBS) and 1% penicillin/streptomycin (Gibco) in a 5% CO₂ humidified atmosphere. Transfection was performed using polyethylenimine (PEI) linear MW 2,500 (Polysciences), following standard protocols.

Generation of Stable Flp-In T-REx HEK293 cell lines

Generation Flp-In T-REx HEK293 cells expressing c-Myc-5-HT_{2A}R-eCFP in an inducible manner was performed following standard protocols³³⁷. Briefly, Flp-In T-REx HEK293 cells were transfected with a mixture containing either c-Myc-5-HT_{2A}R-eCFP cDNA in the pcDNA5/FRT/TO vector and pOG44 vector (1:9) using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. When cotransfected with the pcDNA5/FRT/TO plasmid into the Flp-In mammalian host cell line, the Flp recombinase expressed from pOG44 mediates integration of the pcDNA5/FRT/TO vector containing the gene of interest into the genome through Flp recombination target (FRT) sites. Clones resistant to blasticidin (5.0 µg/ml; InvivoGen) and hygromycin (2.2 µg/ml; InvivoGen) and expressing the Tet repressor (tetR) along with the inserted c-Myc-5-HT_{2A}R-eCFP at the FRT site were screened for eCFP expression by standard fluorescence microscopy protocols.

Double stable cell lines expressing mGluR2-eYFP, mGluR3-eYFP or mGluR2ΔTM4 constitutively and 5-HT_{2A}R-eCFP in an inducible manner were generated from the Flp-In T-REx HEK293 cells described above. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) with the vectors containing mGluR2-eYFP, mGluR3-eYFP or mGluR2ΔTM4. Following transfection, cells were selected for resistance to Geneticin (1 mg/ml; Invitrogen), and the resistant clones were screened for receptor expression by standard fluorescence microscopy. Flp-In T-REx HEK293 cells stably expressing µ-opioid receptor tagged with eYFP and able to express c-Myc-5-HT_{2A}R-eCFP in an inducible manner have previously been described³³⁵. To induce expression of 5-HT_{2A}R-eCFP, cells were treated with doxycycline (DOX) (1.0 µg/ml; 24 – 48 h). Dialyzed FBS was used for cell growth to avoid activation of 5-HT_{2A}R by 5-HT that is routinely present in serum.

Mice

5-HT_{2A}R knockout (*Htr2a*^{-/-}) mice of 129S6/Sv background have previously been described¹⁴⁰. For experiments involving 5-HT_{2A}R^{-/-} mice, wild-type (5-HT_{2A}R^{+/+}) littermates on a 129S6/Sv background were used as controls. Animals were housed at 12 h light/dark cycle at 23°C with food

and water ad libitum. The Institutional Animal Use and Care Committee at Virginia Commonwealth University School of Medicine approved all the experimental procedures.

Cortical primary cultures

Fetuses (E17-18.5) from pregnant mothers were removed using aseptic techniques. Cortical primary cultures were performed as previously reported¹⁴⁰. Cells were maintained for 7 days in vitro before use in experiments.

Immunocytochemistry

In HEK293 cells or mouse cortical primary cultures, immunocytochemical assays were performed as previously reported with minor modifications^{183,251,252}. Briefly, cells were fixed with 4% paraformaldehyde (Sigma) supplemented with 100 μ M CaCl₂ and 100 μ M MgCl₂ for 10 min at room temperature, rinsed with PBS, and washed twice with PBS supplemented with 20 mM glycine. Coverslips were incubated with 0.2% Triton X-100 for 10 min at room temperature and incubated in the dark for 60 min with PBS containing 5% donkey or goat serum (according to the secondary antibody). Primary antibody was added (40 μ l on each coverslip) and incubated overnight at 4°C. Primary antibodies used were rabbit anti-c-Myc (Cell Signaling Technology catalog no. 2272; diluted at 1:250), mouse anti-mGluR2 (Abcam catalog no. 15672; diluted at 6.5 μ g/ml), goat anti-Rab5 (SICGEN catalog no. ab1024; diluted at 6.5 μ g/ml [HEK293 cells] or 5.0 μ g/ml [primary neurons]), rabbit anti-Rab7 (Cell Signaling Technology catalog no. 9367; diluted at 1:100), or rabbit anti-GFP (Invitrogen catalog no. A11122; diluted at 1:500) After washing with PBS, cells were incubated for 60 min at room temperature in the dark with the secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen catalog no. A11011; dilution 1:1000), Alexa Fluor 568 conjugated goat anti-rabbit (Invitrogen catalog no. A11004; dilution 1:1000), Alexa Fluor 568-conjugated donkey anti-goat (Invitrogen catalog no. A11057; dilution 1:1000), or Dylight 650-conjugated donkey anti-mouse (Thermofisher catalog no. SA5-10169 [primary neurons]). For the maturation pathway: ER-Tracker Red (ER-Tr) dying protocol fits mostly manufacturer's recommendations (Thermofisher). Concentration was adjusted to 0.5 μ M of dye in

pre-heated HBSS (Gibco), cells were incubated for 20 minutes in the same conditions as above then fixed with 3% PFA during 2 minutes. For Golgi experiments, fixed cells were permeabilized in a 0.2% Triton X-100/PBS solution then disposed in a PBS/3%FBS/1%BSA blocking solution. Coverslips were then incubated for 60 minutes with 5µg/mL mouse anti-58K primary antibody (Abcam), followed by 50 minutes in 0.5µg/mL conjugated AlexaFluor568 goat anti-mouse IgG secondary antibody (Invitrogen). Nuclei were stained (5 min) with Hoechst 33342 (ThermoScientific). After washing (PBS, 6 × 5 min), the coverslips were mounted on glass slides with Prolong Diamond Antifade Mountant (ThermoFisher).

Confocal microscopy

Fixed cells and living cells were visualized on a Carl Zeiss Axio Observer LSM 710 laser scanning confocal microscope (LSCM) with a Plan-Apochromat 63×/1.40 Oil DIC M27 or 40× Cal objective. Hoechst 33342 was excited by a 405 nm blue diode, and eCFP with a 440 nm laser-pulse. Additionally, 488 nm and 514 nm multi-line Argon lasers were used to excite Alexa Fluor 488, mCitrine and eYFP, respectively, whereas a 561 nm green diode laser was used to excite mCherry and Alexa Fluor 568. Finally, DyLight 650 was excited with a 633 nm HeNe laser line. Emission signals were acquired in the same order using the following Main Beam Splitter/Dichroic Beam Splitter (MBS/DBS) and emission filters (EF) sets: InVis 405/Mirror EF: 410-482nm, InVis 445/Mirror EF: 435-480 nm, 488/Mirror EF: 494-523 nm, 458/514/Mirror EF: 523-543 nm, and 458/561/633/Mirror EF: 591-661 (mCherry) 573-620 (AF568) 654-700 (DyLight650). Pinhole was kept constant and adjusted at 1 airy unit. Living cells (cell cloning) were visualized on a Carl Zeiss Cell Axio Observer Z1 spinning disc confocal microscope (SDCM) with an embedded Yokogawa CSU-X1 spinning disc and a C-Apochromat 63×/1.20 W Korr UV-Vis-IR objective. Illumination sources were a 458 nm and 514 nm (multi-Ar) laser for eCFP and eYFP, respectively. Emission filters (SD mirror + bypass filters BP) were RTFT457/514/647 + BP485/30 (eCFP) and

RQFF405/488/568/647 + BP525/50 (eYFP). During image acquisition, cells were maintained at 37°C and 5% CO₂ atmosphere by an incubation system.

Immunoblot Assays

Western blot experiments were performed as previously reported²⁵², using rabbit anti-c-Myc (Cell Signaling, catalog no. 2272; diluted at 1:400) and rabbit anti-β-actin (Abcam catalog no. 8227; diluted at 0.33 μg/ml).

TAT-fused transmembrane domain interference peptides

Peptides derived from human mGluR2 TM4 and TM1 were custom synthesized (Genemed Synthesis, Inc.). TAT (trans-activating transcriptional activator from human immunodeficiency virus; YGRKKRRQRRR) was fused to the N-terminus of the human mGluR2 TM4 (YGRKKRRQRRRSPASQVAICLALISGQLLIVVAWLVE) and TM1 (AWAVGPVTIACLGALATLFLVGVFVRHYYGRKKRRQRRR) to obtain the correct orientation for the inserted peptide because TAT binds to phosphatidylinositol-(4,5)-biphosphate found on the inner surface of the membrane³³⁸. TAT-labelled 38-mer peptide was used as an internal control (YGRKKRRQRRR-VIAPLYTSCVNWQIF-VISMARGARVAI). Stock solutions of the TAT-tagged peptides (5.2 mM) were prepared in DMSO supplemented with glacial acetic acid (1.93%). For FRET assays, cells were incubated with TAT-tagged peptides (10 μM, final concentration) for 60 min (DMSO's final concentration = 0.18%). For studies involving receptor agonists, TAT-tagged peptides were added 5 minutes before DOI or LY379269, or vehicle administration. For studies involving overnight treatment with clozapine, TAT-tagged peptides were added both 5 minutes before clozapine or vehicle administration and 65 minutes before cell fixation.

Proximity Ligation Assay

Heteromers of c-Myc-5-HT_{2A}R-eCFP and mGluR2-eYFP were detected using the Duolink In Situ PLA Detection Reagents Red technology kit (Sigma-Aldrich). Briefly, coverslips with fixed cells were incubated with 0.1% Triton X-100 for 10 min at room temperature then in blocking solution

according to manufacturer's instructions. Cells were incubated overnight at 4°C with a mixture of the primary antibodies (rabbit anti-c-Myc [Cell Signaling Technology, catalog no. 2272; 1:250], and mouse anti-mGluR2 [Abcam, catalog no. 15672; 1:200]). Samples were incubated with Duolink In Situ PLA Probe Anti-Rabbit PLUS Affinity purified Donkey anti-Rabbit IgG and Duolink In Situ PLA Probe Anti-Mouse MINUS Affinity purified Donkey anti-Mouse IgG. Samples were processed for ligation and amplification and were mounted onto glass slides with Prolong Diamond Mountant. Z-stack images were acquired in a Carl Zeiss Axio Observer LSM 710 laser scanning confocal microscope with a Plan-Apochromat 63×/1.40 Oil DIC M27 objective (to obtain a representative image) or 40× Cal objective (for quantification purposes). For each field of view, a stack of three channels (one per staining) was obtained. Images were processed with Fiji software version 2.0.0 (National Institutes of Health, Bethesda, MD). To quantify PLA signal, PLA-red channel remained hidden during cell identification process. Automated counting involved selection and cropping of each region of interest, Z-Stack projection of the PLA channel, subtraction of background with a rolling ball radius of 20 pixels and finding maxima with a noise tolerance value of 6000–10000 depending on the experiment and the background.

Radioligand binding assays

[³H]Ketanserin, [³H]LY341495 and [³⁵S]GTP γ S binding assays were performed as previously reported^{183,251,252}. The stoichiometry of 5-HT_{2A}R density (as assessed by [³H]ketanserin binding) and mGluR2 density (as assessed by [³H]LY341495 binding) is similar to that previously observed in native tissue, such as mouse frontal cortex and postmortem human frontal cortex^{183,251,252} and in cross-talk positive clones²⁵⁴.

[Ca²⁺]_i Mobilization assays

Two days before the assay, cells were plated onto poly-D-lysine coated 96-well plates (Greiner Bio-One GmbH) and, 48 hours later, treated with doxycycline (1 μ M) for 40 h. Cells were washed with DPBS and loaded with 3 μ M Fura 2-AM (Molecular Probes) in imaging solution (5 mM KCl,

0.4 mM KH₂PO₄, 138 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM CaCl₂, 1 mM MgCl₂, 6 mM glucose, 20 mM HEPES, pH 7.4) supplemented with pluronic acid (20% solution in DMSO). After incubation for 30 min at 37°C, cells were washed twice with washing buffer before being placed on the FlexStation 3 microplate reader (Molecular Devices). The Fura-2 signal was acquired at 510 nm by switching the excitation wavelength between 340/380 nm. Intracellular calcium concentration was expressed as a 340/380 nm ratio, and values were normalized to the basal 340/380 nm ratio recorded during 30 seconds before perfusion of the drug using Softmax Pro (Molecular Devices). To allow efficient coupling of G_{α_{i/o}}-coupled mGluRs to the PLC pathway, cells were also transfected with the chimeric G_α protein G_{α_{q19}}³³⁶. Concentrations of LY379268 were selected based on our previous findings²⁵¹. Thus, activation of the canonical G_{α_{i/o}} protein-dependent pathway was tested in the presence of LY379268 (0.1 μM), but trans-activation of the G_{α_{q11}} protein-dependent pathway was tested in the presence of LY379268 (10 μM and 100 μM).

Flow cytometry and immunofluorescence

Cells were treated with for 60 min with either DOI (1 μM), LY379268 (10 μM), or vehicle, or (overnight) with clozapine (10 μM) M100907 (10 μM) or vehicle. Cells were trypsinized, washed with PBS, filtered through filter top tubes (35 μm), resuspended in PBS supplemented with 1% FBS, and analyzed using a LSRFortessa X-20 Flow cytometer (BD Bioscience). eCFP and eYFP were excited with a 405 nm and a 488 nm laser line, respectively. Emission filters were 525/50 and YFP-A. Quantification was carried out using FCS Express v.5 (De Novo Software). Mean intensity values were determined by gating 25.5% or more of the total cell population. Imaging flow cytometry (Amnis ImageStreamX) to measure colocalization of mGluR2 or 5-HT_{2A}R with the transferrin receptor at the single cell level was performed as previously reported, using an Amnis ImageStream flow cytometer²⁵⁴. Cells were considered to present a colocalized signal between the receptor constructs and transferrin receptor when their Bright Detail Similarity score was greater than 2. To detect cell surface receptors, cells were treated with DOI, LY379268 or vehicle

(60-min treatment), or with clozapine, M100907 or vehicle (overnight treatment). Cells were washed with ice-cold imaging buffer (PBS supplemented with 10% FBS) and incubated with human mGluR2 Alexa Fluor 647-conjugated antibody (R&D Systems, catalog no. IC4676R; 1:40) for 60 min on ice (~11-13°C). After two washes with ice-cold imaging buffer, cells were resuspended in ice-cold imaging buffer and loaded into the flow cytometer. 405 nm, 488 nm and 640 nm lasers were used to excite eCFP, eYFP and AF647, respectively. Data were subsequently gated and analyzed using the FCS Express 5 software. For the maturation pathway: IFC assays were performed on the Amnis ImageStreamX Mark II flow cytometer (IS). Prior to analysis, probe-free cells and single color channels controls were made in order to evaluate background autofluorescence and to set the compensation matrix parameters required to separate spectral overlap of fluorescent probes. For BiFC experiments, cells were transfected 24h before experiment on 10 cm plates using scaled up DNA/PEI concentrations. mCitrine and ER-Tr were illuminated with a 488 nm and a 561 nm lasers respectively. In-focused cells were acquired with a 60x objective and gated by gradients MAX and RMS controlling bright field focus quality, then proceeded to similarity analysis. The figure herein displays

an example of negative control performed on the 5-HT_{2A}R BiFC. Cells populations were gated by calculating the maximal threshold rendered from irrelevant colocalization of each channel with the bright field (>1.5) in ImageStream Data Exploration and Analysis Software (IDEAS). The BDS

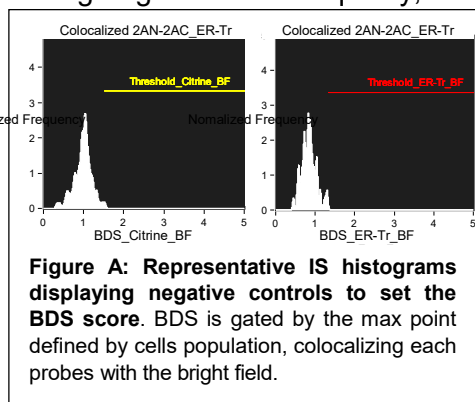


Figure A: Representative IS histograms displaying negative controls to set the BDS score. BDS is gated by the max point defined by cells population, colocalizing each probes with the bright field.

score is then determined and set to gate and identify highly colocalized populations²⁵⁴. BDS is a log-transformed Pearson Correlation Coefficient.

SensorFRET

SensorFRET assays were performed as previously reported³³⁹. Briefly, the fluorophores associated with uneven expression levels between the donor and acceptor. The fluorophores used as the FRET pair were eCFP (donor) and eYFP (acceptor) with quantum yields assumed to be

0.40 and 0.61, respectively³⁴⁰. This approach also requires knowledge of the fluorophore emission shapes (for linear unmixing of the spectra) as well as the relative molar extinction coefficients for the fluorophores at each excitation frequency (characterized by the γ parameter). The normalized emission shapes of both fluorophores as well as the autofluorescence at each excitation frequency were determined experimentally. The γ calibration term was also determined experimentally ($\gamma = 0.114$), following a previously described procedure³³⁹. Imaging was carried out using a Zeiss 710 confocal LSM at 405nm and 458 nm excitation frequencies and a 32-channel spectral detector spanning an emission range of 416nm to 718nm. In unfixed cells, short acquisition times (~ 600 ms) were necessary to minimize the shift due to vesicle movement during the time needed to acquire images at both excitation wavelengths. For assays involving TAT-tagged peptides, cells were briefly fixed with PFA (1%) for 5 min to avoid background caused by PFA fixation. A minimum of 30 cells were imaged for each experimental group. For the statistical analysis, the measured FRET efficiency of each vesicle was considered a single observation. Strict masking was carried out to include vesicles that met three criteria. First, only vesicles which remained stationary between the 405 and 458 nm images were included because even at short acquisition times, some population of vesicles showed appreciable movement. Second, only vesicles with a donor to acceptor stoichiometry within the range of 1:10 to 1:1 were included because the sensorFRET approach is not applicable when there are excess donor fluorophores³³⁹. Thus, below a 1:10 ratio the donor signal is so small that it is difficult to reliably distinguish it from auto fluorescence and noise. The stoichiometry was estimated by comparing the ratio of the donor and acceptor direct excitation in experimental vesicles to a unimolecular construct (both CFP and YFP are encoded as part of the same protein) where the stoichiometry is known to be 1:1. Marks were drawn only over pixels that showed colocalized eCFP and eYFP signal from both 405 nm and 458 nm images. Finally, only vesicles consisting of greater than 5 pixels were included so that mis-localization of proteins to small non-vesicle features was excluded, allowing each vesicle observation to be the average of multiple spectra and improving the signal to noise ratio.

Electron Microscopy

Electron microscopy assays were carried out as previously reported with minor modifications²⁵². Briefly, four male mice (8 – 12 weeks old) per genotype were deeply anesthetized and perfused transcardially with 2% dextran (MW 70,000) in 0.1 M phosphate buffer (PB, pH 7.4), and then with a mixture of 4% paraformaldehyde and 0.125% glutaraldehyde in phosphate buffered saline (PBS). Brains were removed and post-fixed in the same perfusate overnight at 4°C. Approximately 0.5-mm-thick coronal slices were dissected from the regions of interest. Tissue blocks through superficial layers of prefrontal cortex were embedded in Lowicryl HM20 resin (Electron Microscopy Sciences, Fort Washington, PA) by freeze substitution and low-temperature embedding of the specimens using methods previously described^{341–343}. Briefly, slices were cryoprotected by immersion in 4% D-glucose, followed with increasing concentrations of glycerol (from 10% to 30% PB). Sections were plunged rapidly into liquid propane cooled by liquid nitrogen (–190°C) in a Universal Cryofixation System KF80 (Reichert-Jung, Vienna, Austria). The samples were immersed in 0.5% uranyl acetate dissolved in anhydrous methanol (–90°C, 24 h) in a cryosubstitution AFS unit (Leica, Vienna, Austria). The temperature was raised from –90°C to –45°C in steps of 4°C/h. After washing with anhydrous methanol, the samples were infiltrated with Lowicryl HM20 resin at –45°C. Polymerization with ultraviolet light (360 nm) was performed for 48 h at –45°C, followed by 24 h at 0°C. Ultrathin sections (80 nm) were cut using a diamond knife on a Leica UC7 ultramicrotome, and mounted on 300 mesh nickel grids (EMS) coated using a Coat-Quick adhesive pen (EMS). Grids with sections were incubated in 0.1% sodium borohydride and 50 mM glycine for 6 minutes at room temperature, washed and incubated for 10 min in Tris-buffered saline with 0.1% Triton X-100 (TBST) containing 2% albumin. For immunolabeling, sections were incubated overnight in primary anti-mGluR2 antibody (Abcam, mouse monoclonal antibody catalog no. 15672, 10 µg/ml) in the same diluent as above, washed with TBST, and incubated with secondary anti-goat 10 nm gold-tagged antibody (EMS; 1:20) in TBST (2% albumin and polyethyleneglycol 20,000; 5 mg/ml) for two hours, washed and counter-stained with 1%

uranyl acetate (aq.). The specificity of the primary antibody against mGluR2 was previously confirmed in experiments with knockout mice²⁵². Grids were viewed on a Hitachi 7000 transmission electron microscope and imaged at a final captured image magnification of 130K. Imaging concentrated on deep layer 2-3 of the prefrontal cortex. Excitatory (asymmetric) glutamatergic synapses were identified by the presence of a thickened postsynaptic density, synaptic cleft, and clusters of round presynaptic vesicles³⁴⁴. A synapse was scored as immunolabel-positive if it contained at least 3 gold particles in the synaptic compartments described above, and 25-35 labeled synapses were imaged per animal/group. Overall, ~90% of labeled synapses showed gold particles associated within the postsynaptic element, though a small population of synapses contained gold particles limited to its presynaptic compartment. Gold particle distribution with respect to plasmalemma was scored for each postsynaptic site as membrane only, intracellular only, or both. Membrane labelling was defined by gold particles falling within 25 nm of the plasmalemma (a distance defined by the approximate size of the primary and secondary antibodies).

Data acquisition and statistical analysis

Statistical analysis was performed with a GraphPad Prism software version 8. Immunocytochemical assays were acquired using confocal fluorescence microscopy at identical settings for each of the experimental conditions. For colocalization assays, raw 16-bit image files (.czi) were imported into the Carl Zeiss ZEN software (Black version) and/or Fiji version 2.0.0 with Coloc 2 plugin. To assess colocalization, Pearson's and/or Manders' coefficients were calculated because these colocalization coefficients are based on pixel-intensity-correlation measurement and do not include object-recognition approaches. Pearson's coefficient, which ranges from +1 (total positive linear correlation) to -1 (total negative correlation; 0 defines absence of linear correlation) is sensitive to both signal cooccurrence and the more rigorous condition of signal correlation (pixel-for-pixel proportionality in the signal levels of the two channels). However, unlike Pearson's correlation coefficient, Manders' fractional overlap coefficients (M1 and M2), which

measure the fraction of a probe in a probe-tagged compartment and range from 0 (no overlap) to 1 (perfect overlap), strictly measures co-occurrence independent of signal proportionality^{345,346}. Colocalization of two fluorescently labelled 7TMR constructs (5-HT_{2A}R-eCFP and mGluR2-eYFP) was assessed both by Manders' and Pearson's coefficients, whereas colocalization of a fluorescently labelled 7TMR construct with a marker of endosomal vesicles (Rab5) was assessed by Manders' coefficient. Both Manders' and Pearson's coefficients within the region of interest were calculated after setting single color thresholds by either colocalizing single probe channels or manually defining a threshold mask upon background subtraction in all channels using Rolling Ball Radius of the largest object in the image (~ 20 – 30 μm). Colocalization was scored in at least 25 cells per experimental condition in three independent assays where the experimenter was blinded to the experimental conditions. For Manders' and Pearson's coefficients, cell regions of interest were demarcated according to the eYFP signal located at the plasma membrane as a potential cell surface marker. For FRET signal, pixels of interest were demarcated based on their intracellular colocalization of eCFP and eYFP signal. For PLA signal, cell regions of interest were demarcated according to eYFP signal within previously defined eCFP-positive cells. For intracellular eYFP signal, cell regions of interest were randomly demarcated based on eCFP signal within intracellular vesicles. Signal intensity for intracellular eYFP was assessed with the Carl Zeiss ZEN software (Black version). The laser intensity was kept constant among all experimental conditions. Straight-line selections were drawn from the plasma membrane to the nucleus or across the cytoplasm, and pixel intensities across the line were measured using plot profile in Fiji. Pixels with maximum and minimum intensities were normalized to 100 and 0, respectively. The statistical significance of experiments involving three or more groups and two or more treatments was assessed by two-way ANOVA followed by Bonferroni's post hoc test. Statistical analysis of experiments involving three or more groups was assessed by one-way ANOVA followed by Bonferroni's post hoc test. Statistical analysis involving two groups was assessed by Student's *t*-test. For FRET analysis, statistical analysis was assessed by Mann-Whitney U test or one-way

non-parametric ANOVA (Kruskal-Wallis) with Dunn's post hoc test because FRET datasets show a Cauchy distribution with heavier tails. Normality and homoscedasticity assumptions were checked prior to any calculation. Radioligand binding saturation curves were analyzed using a nonlinear curve fitting approach. An extra-sum-of-squares (F test) was used to determine statistical significance for simultaneous analysis of binding saturation curves and [^{35}S]GTP γ S binding assays. The level of significance was set at $P = 0.05$. Values included in the figure legends represent mean \pm SEM, except for FRET assays where figure legends represent median with 95% confidence interval.

Results

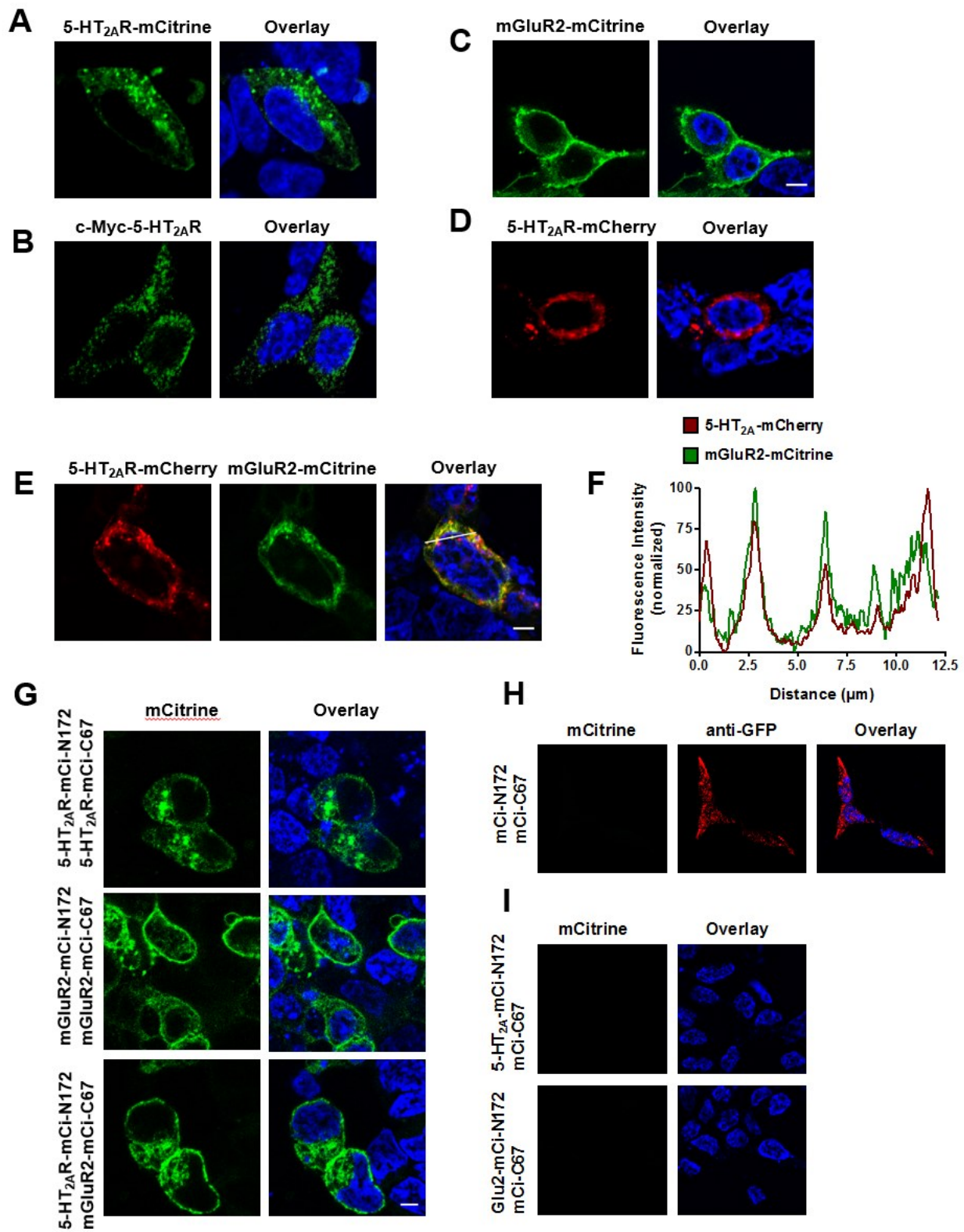
I. Cellular distribution of mGluR2 and 5-HT_{2A}R alone and together in HEK293 cells

1. 5-HT_{2A}R distribution pattern in HEK293 cells

To first determine and compare expression patterns of human mGlu2 and 5-HT_{2A} receptors in a heterologous cellular system and to validate previous findings showing an elevated intracellular localization of the 5-HT_{2A}R, we introduced the mCitrine sequence at the C-terminus to generate 5-HT_{2A}R-mCitrine. As shown before^{251,347}, HEK293 cells transfected with the 5-HT_{2A}R-mCitrine exhibit a fluorescent signal mostly localized in intracellular vesicles (Fig. 1A). To rule out a direct effect of the C-terminally tagged mCitrine on receptor trafficking, we used a second method where the epitope c-Myc was tagged to the N-terminus of the 5-HT_{2A}R construct. Similar to the distribution pattern observed with 5-HT_{2A}R-mCitrine, the c-Myc-5-HT_{2A}R construct was primarily located intracellularly (Fig. 1B).

2. Cotransfection of 5-HT_{2A}R and mGluR2

mGlu2 receptor exhibits a standard surface receptor distribution where most of the expression is visible at the plasma membrane area (Fig. 1C). However, when 5-HT_{2A}R and mGluR2 constructs were co-transfected, mGluR2 pattern of expression was dramatically changed. In contrast to the subcellular localization observed in HEK293 cells transfected with the mGluR2-mCitrine construct alone, coexpression of 5-HT_{2A}R-mCherry led to the partial internalization of mGluR2-mCitrine (Figs. 1, D and E), which can also be demonstrated by a high level of colocalization of mGluR2-mCitrine and 5-HT_{2A}R-mCherry in intracellular vesicles (Fig. 1F). Additionally, not all the cotransfected cells exhibited the observed relocation of mGluR2, not in the same proportion. This suggests that the stoichiometry can affect the change responsible for the mGluR2 subcellular distribution.



(Fig 1)

Fig. 1. 5-HT_{2A}R affects localization of mGluR2. (A and B) Representative confocal micrographs of HEK293 cells transfected to express 5-HT_{2A}R-mCitrine (A) or c-Myc-5-HT_{2A}R (B). Nonpermeabilized cells were imaged to detect mCitrine (A), whereas permeabilized cells were stained with anti-c-Myc and secondary antibody, and imaged to detect anti-c-Myc (B). (C to E) HEK293 cells were transfected to express mGluR2-mCitrine alone (C), 5-HT_{2A}R-mCherry alone (D), or 5-HT_{2A}R-mCherry and mGluR2-mCitrine (E). (F) Representative line scan. (G) BiFC signal in HEK293 cells transfected to coexpress 5-HT_{2A}R-mCi-N172 and 5-HT_{2A}R-mCi-C67, mGluR2-mCi-N172 and mGluR2-mCi-C67, or 5-HT_{2A}R-mCi-N172 and mGluR2-mCi-C67. (H and I) Representative micrographs of BiFC controls. HEK293 cells co-transfected to express mCi-N172 and mCi-C67 (probes only) and submitted to immunofluorescence assay using antibody against GFP conjugated to a fluorescent secondary antibody (H). Absence of mCitrine signal when cells were cotransfected either with both 5-HT_{2A}R-mCi-N172 and mCi-C67 or with both mGluR2-mCi-N172 and mCi-C67 (I). Nuclei were stained in blue with Hoechst. Scale bars, 5 μ m.

II. Intracellular localization of the 5-HT_{2A}R-mGluR2 heterocomplex

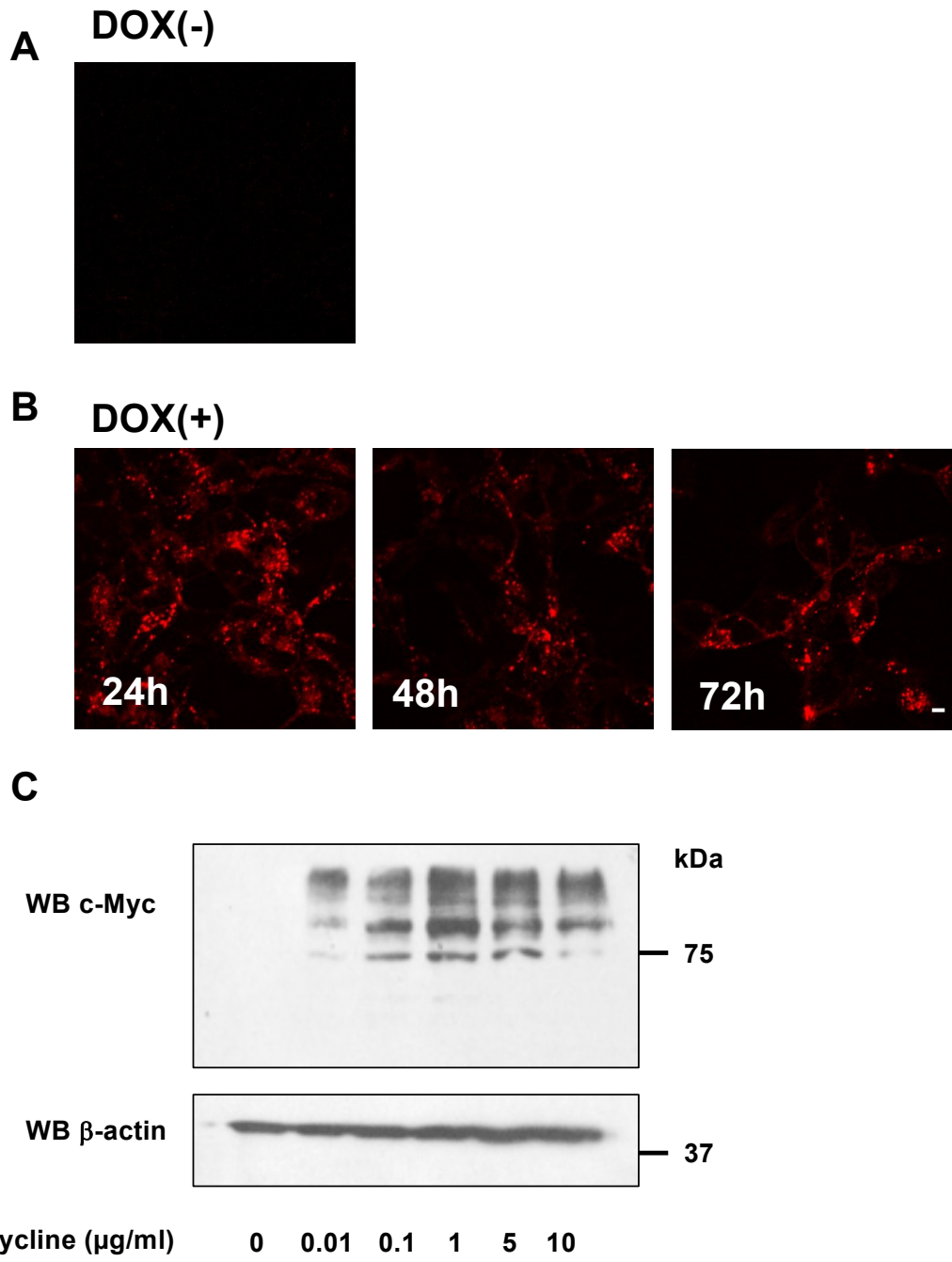
1. Intracellular complementation of 5-HT_{2A} and mGluR2

The 5-HT_{2A}-mGluR2 receptor heteromer study resembles a niche in the field of 7TMRs oligomers. Our laboratory has become an expert in the study of the structure and function of this heterocomplex. We thus expected to locate it at the cell boundaries, using several RRI assays. To track the intracellular physical interaction between 5-HT_{2A}R and mGluR2 in HEK293 cells, we used bimolecular fluorescence complementation (BiFC) as a biophysical technique that allows visualization of protein complexes in transfected mammalian cells^{348,349}. BiFC enables generation of a single fluorescent signal when each of the constructs fused to a fragment of the mCitrine fluorescent protein interact. The recovery of the protein quaternary structure attests the interaction between the two proteins. BiFC signal was detected in cells cotransfected with 5-HT_{2A}R fused to either the N-terminal 172 amino acid fragment or the C-terminal 67 amino acid fragment of mCitrine (mCi-N172 and mCi-C67, respectively) (Fig. 1G), which further corroborates the capability of 5-HT_{2A}R to form a family A GPCR homodimer¹⁶⁵. Additionally, BiFC signal in cells coexpressing 5-HT_{2A}R-mCi-N172 and 5-HT_{2A}R-mCi-C67 was observed intracellularly (Fig. 1G), whereas BiFC signal in cells coexpressing mGluR2-mCi-N172 and mGluR2-mCi-C67 was mostly detected at the plasma membrane (Fig. 1G). Importantly, this pattern of BiFC signal was redistributed in cells coexpressing 5-HT_{2A}R-mCi-N172 and mGluR2-mCi-C67 (Fig. 1G), which suggests that physical interaction between 5-HT_{2A}R and mGluR2 can be detected both close to the cell surface as well as intracellularly. Internal controls to validate BiFC signal specificity included cotransfection of mCi-N172 and mCi-C67 constructs (Fig. 1H), as well as mCi-C67 and 5-HT_{2A}R-mCi-N172, or mCi-C67 and mGluR2-mCi-N172 (Fig. 1I).

III. GPCR heteromerization is necessary for the 5-HT_{2A}R-mediated mGluR2 internal relocation

The use of stable cellular systems as a reductionist approach to observe trafficking events after receptor activation was chosen as a model for this study. This choice has several advantages, including overcoming expression variability and transfection efficiency issues – it is easy to routinely grow, treat and analyze, and to manage the expression levels. To that purpose, we devised an experimental Flp-In T-REx HEK293 cellular system that enables small molecule doxycycline (DOX)-inducible expression of the inserted human *HTR2A* gene construct along with the stable expression of mGlu2, mGlu3 or mGlu2ΔTM4 (see below) receptors. The Flp-In T-REx system allows expression of the inserted construct into the cell genome via Flp-In recombinase-mediated DNA recombination at the Flp recombination target (FRT) site, which can be controlled by the incorporation DOX to the cell culture medium. This model of stable cell lines has already been employed to great advantage in the study of receptor pharmacology, trafficking and oligomerization^{45,335}. Here we used Flp-In T-REx HEK293 cells to further explore the effect of 5-HT_{2A}R on subcellular localization of mGluR2.

The c-Myc-5-HT_{2A}R-eCFP construct was inserted into the appropriate locus of the Flp-In expression system, and pools of positive Flp-In T-REx HEK293 cells were selected. No visible expression of the eCFP-tagged construct was observed in the absence of DOX (Fig. 2A). However, presence of DOX resulted in expression of the integrated construct as monitored by fluorescence corresponding to eCFP (Fig. 2B) and immunoblotting with anti-c-Myc antibody (Fig. 2C), which also serves as a concentration indicator of receptor expression. Induction of expression of c-Myc-5-HT_{2A}R-eCFP was concentration- and time-dependent, reaching maximal levels within 30 – 40 h upon 1 μg/mL [DOX] administration (Figs. 2, B and C), and lasted a couple of days, approximately. Additionally, as shown above in HEK293 cells transiently transfected with the 5-HT_{2A}R construct, visualization of living Flp-In T-REx HEK293 cells corroborated that the bulk of c-



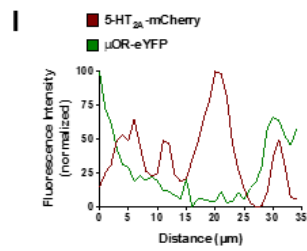
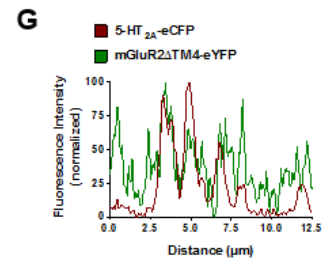
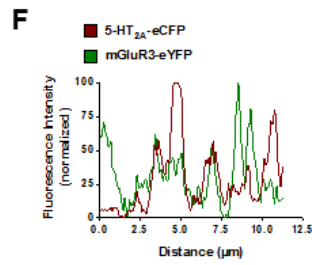
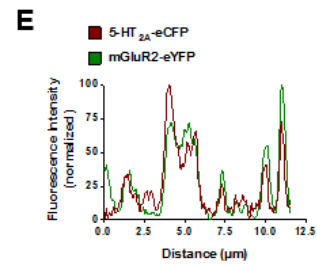
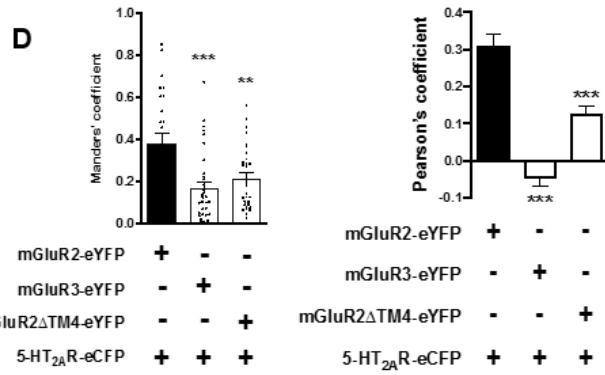
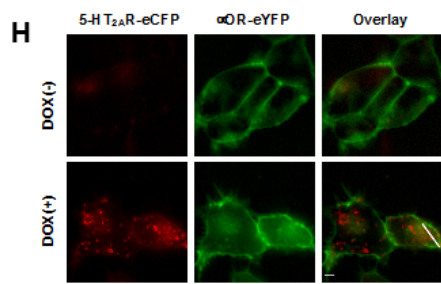
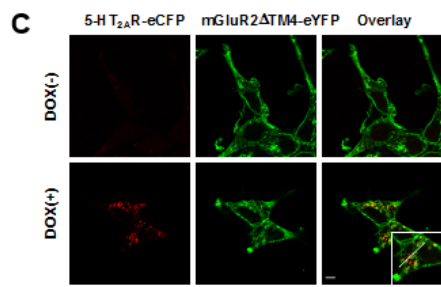
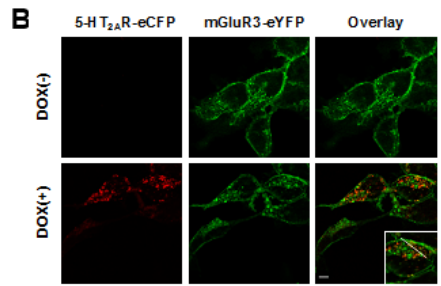
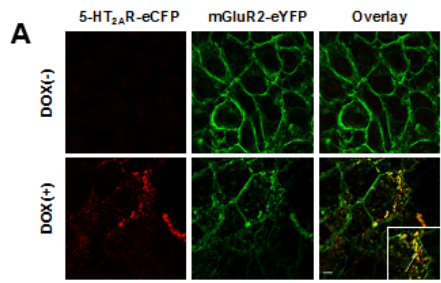
(Fig. 2)

Fig. 2. Confocal micrographs of Flp-In T-REx HEK293 cells expressing the 5-HT_{2A} receptor. (A and B) Representative live-cell confocal micrographs of Flp-In T-REx HEK293 cells harboring c-Myc-5-HT_{2A}R-eCFP at the inducible locus. Cells were left untreated [DOX(-)] (A) or treated for 24h, 48h, or 72 h with doxycycline [DOX(+)] (B). (C) Membranes from Flp-In T-REx HEK293 cells harboring c-Myc-5-HT_{2A}R-eCFP at the inducible locus left untreated or treated with doxycycline at the indicated concentration were immunoblotted with anti-c-Myc (upper panel) or anti-β-actin. Scale bar, 5 μm.

Myc-5-HT_{2A}R-eCFP was located intracellularly (Fig. 2B). Those experiments generated a series of parental clones expressing inducible 5-HT_{2A}R.

In order to demonstrate the involvement of 7TMR heteromerization in the relocalization of mGluR2 following its coexpression with 5-HT_{2A}R, we first based our negative controls with mGluR3 as well as with an mGluR2/mGluR3 chimeric construct that does not form a receptor heteromer with 5-HT_{2A}R. We selected the mGluR2/mGluR3 chimeric construct mGluR2 Δ TM4 because, according to previous findings from substitution of residues Ala-677^{4.40}, Ala-681^{4.44} and Ala-685^{4.48} in mGluR2 for Ser-686^{4.40}, Phe-690^{4.44} and Gly-694^{4.48} in mGluR3 decreases signal that indicates heteromeric assembly. This was demonstrated based on the use of several independent experimental approaches, including flow cytometry-based fluorescence energy resonance transfer (FCM-based FRET), FRET microscopy, and coimmunoprecipitation in HEK293 cells^{251,252}.

The parental c-Myc-5-HT_{2A}R-eCFP expressing clones were then transfected with either mGluR2-eYFP, mGluR3-eYFP or mGluR2 Δ TM4-eYFP, and individual clones constitutively eliciting eYFP signal were selected. Visual inspection of eYFP fluorescence confirmed that, in the absence of DOX, mGluR2-eYFP was localized at the cell boundaries (Fig. 3A). Remarkably, DOX-induced expression of 5-HT_{2A}R-eCFP dramatically affected the subcellular distribution of mGluR2-eYFP, now partially internalized in vesicles (Fig. 3A). This effect of 5-HT_{2A}R-eCFP expression was not observed in cells stably expressing either mGluR3-eYFP (Fig. 3B) or mGluR2 Δ TM4-eYFP (Figs. 3C). Both mGluR3-eYFP and mGluR2 Δ TM4-eYFP showed a higher degree of intracellular localization as compared to mGluR2-eYFP, yet both quantitative evaluation (Fig. 3D) and representative line scans (Figs. 3, E to G) demonstrated significantly less subcellular proximity between 5-HT_{2A}R-eCFP and mGluR3-eYFP or mGluR2 Δ TM4-eYFP, as compared to the high degree of overlap between 5-HT_{2A}R-eCFP and mGluR2-eYFP. The subcellular distribution of the class A G $\alpha_{i/o}$ protein-coupled μ -opioid receptor, which is a prototypical plasma membrane-



(Fig 3)

Fig. 3. Effect of 5-HT_{2A}R on localization of mGluR2 requires heteromerization. (A to I) Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP (A, D and E), mGluR3-eYFP (B, D and F), mGluR2 Δ TM4-eYFP (C, D and G), or μ OR-eYFP (H and I) and harboring 5-HT_{2A}R-eCFP at the inducible locus were untreated [DOX(-)] or treated with doxycycline [DOX(+)]. Representative confocal micrographs (A to C and H) and corresponding line scans (E to G and I). (D) Manders' and Pearson's colocalization coefficients analysis of eYFP- and eCFP-tagged constructs (n = 25 – 33 cell regions of interest in three independent experiments). Data are mean \pm SEM (D). Statistical analysis was performed using the one-way ANOVA (D) with Bonferroni's *post hoc* test. ***P* < 0.01, ****P* < 0.001. Scale bars, 5 μ m.

localized 7TMR, was not evidently affected by DOX-induced expression of 5-HT_{2A}R-eCFP (Figs. 3, H and I), further comforting the specificity of the relation between 5-HT_{2A}R and mGluR2.

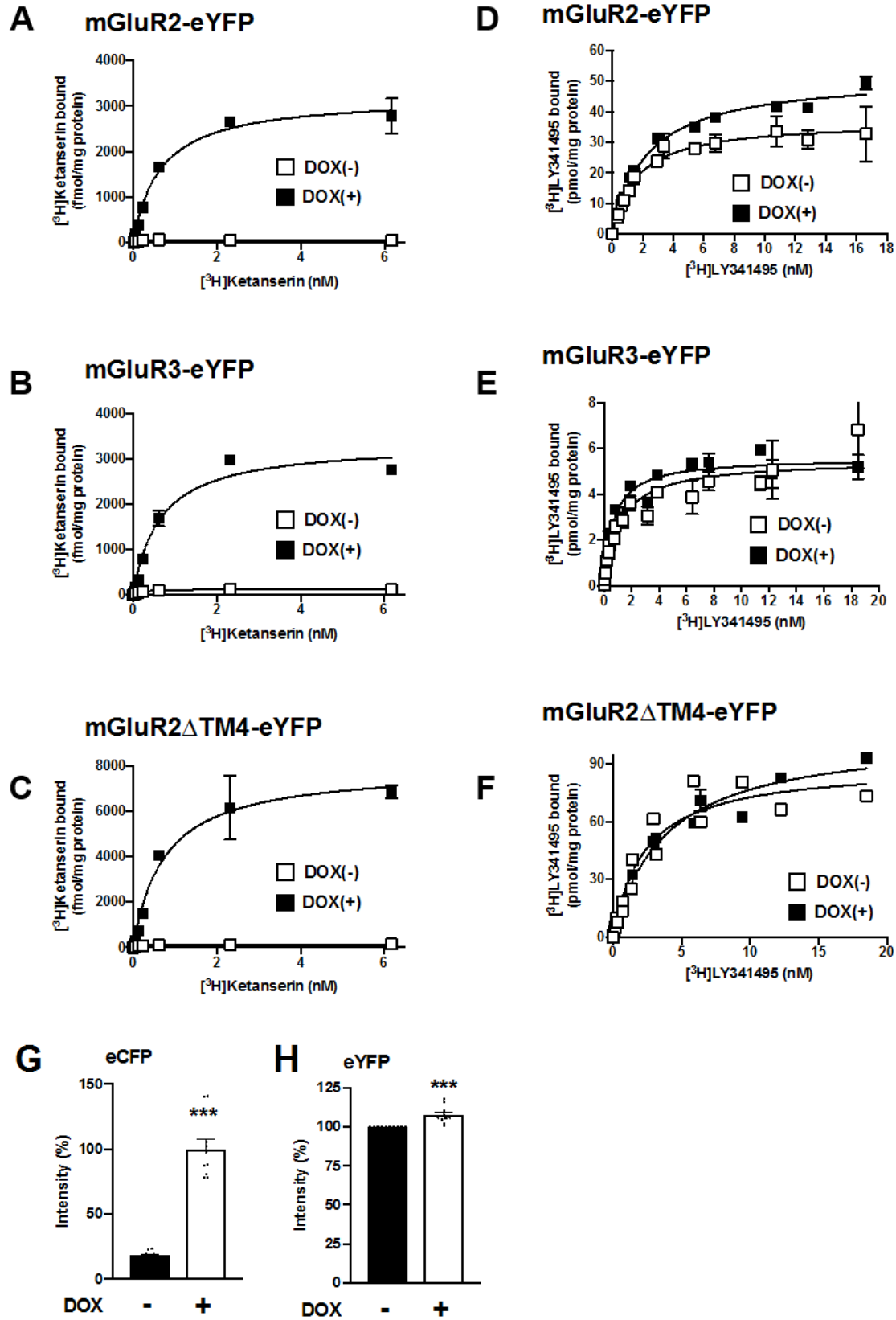
IV. Characterization of the double stable cells lines

Radioligand binding assays with the 5-HT_{2A}R antagonist [³H]ketanserin confirmed that addition of DOX resulted in 5-HT_{2A}R-eCFP expression in cells stably expressing mGluR2-eYFP, mGluR3-eYFP or mGluR2ΔTM4-eYFP (Figs. 4, A to C, and Table 1). Using the mGluR2/3 antagonist [³H]LY341495, we also observed that DOX-induced expression of 5-HT_{2A}R-eCFP led to a statistically significant increase in the density of mGluR2-eYFP, an effect that was not observed in cells stably expressing mGluR3-eYFP or mGluR2ΔTM4-eYFP (Figs. 4, D to F, and Table 1). This effect of DOX-induced 5-HT_{2A}R-eCFP expression on augmentation of mGluR2-eYFP density was corroborated by fluorescence flow cytometry assays (Figs. 4, G and H).

We next assayed the effect of the mGluR2/3 agonist LY379268 on stimulation of [³⁵S]GTPγS binding as a functional readout of receptor-Gα_{i/o} protein coupling. Interestingly, DOX-induced expression of 5-HT_{2A}R-eCFP reduced the functional properties of LY379268 augmenting [³⁵S]GTPγS binding in cells stably expressing mGluR2-eYFP (Fig. 4I), an allosteric event that was absent in cells stably expressing mGluR2ΔTM4-eYFP (Fig. 4J). Although activation of mGluR2 elicited a robust G protein signal after ligand binding, we were unable to test mGluR3 function with the [³⁵S]GTPγS assay on clones stably expressing mGluR3. In order to overcome this issue, Gα_{i/o}-coupled receptors mGluR (*i.e.*, mGluR2, mGluR3 and mGluR2ΔTM4) ability to trigger the phospholipase C pathway following activation of the G Protein, we transfect DOX(-) cells with the chimeric G protein Gα_{q19} and analyzed the fold increase in [Ca²⁺]_i (Fig. 4K) following application of 0.1 μM of the mGluR2/3 agonist LY379268. Together with the effects of 5-HT_{2A}R on mGluR2 subcellular redistribution (see above), these findings suggest that, although DOX-induced expression of 5-HT_{2A}R results in augmentation of total mGluR2 density, its functional properties for agonist-induced Gα_{i/o} protein recruitment are diminished.

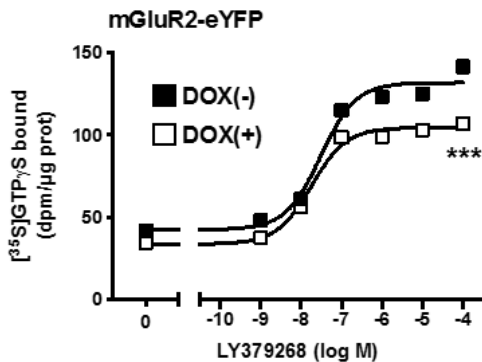
Activation of 5-HT_{2A}R stimulates the β -isoforms of phospholipase C (PLC- β) that catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP₂), leading to the production of two second messengers, namely inositol triphosphate (IP₃) and diacylglycerol (DAG). The G α_q -mediated signal is then transmitted through to those molecules: IP₃ binds to its ER localized receptor and open the channel allowing mobilization of the internal store and efflux of calcium in the cytosol. DAG binds to the plasma membrane to recruit the protein kinase C that is activated by Ca²⁺ and mediates several other pathways. Our previous results suggested that stimulation of cells coexpressing mGluR2 and 5-HT_{2A}R with the mGluR2/3 selective agonist LY379268 leads to a G $\alpha_{q/11}$ protein-dependent increase in the concentration of [Ca²⁺]_i^{251,253,254}, a transactivation mechanism event that has been validated by some^{170,350} but not all studies³⁵¹.

Besides physically interacting, 5-HT_{2A}R and mGluR2 have been shown to crosstalk by modulating their respective G proteins signaling. The endogenous ligands serotonin and glutamate tend to increase the G α_i signaling and reduce the G α_q signaling within the heterocomplex. Intracellular calcium mobilization mediated by inositol 1,4,5-triphosphate from endoplasmic reticulum compartments can be monitored by calcium-sensitive fluorescent dyes. The highly sensitive ratiometric calcium dye Fura-2 was used to assess calcium elevation in our clones expressing mGluR2 or mGluR2 Δ TM4-eYFP along with the induction of 5-HT_{2A}R expression. Application of 10 μ M of LY379268 was sufficient to increase the intracellular calcium concentration in cells expressing mGluR2, with a much lesser signal for the cells expressing the mutant receptor mGluR2 Δ TM4-eYFP. This effect seemed dose-dependent as with 100 μ M an even greater signal was observed (Figs. 4, L and M). As expected, neither serotonin (5-hydroxytryptamine, 5-HT) nor LY379268 had such an effect on Ca²⁺ release in DOX(-) Flp-In T-REx HEK293 cells, whereas 5-HT stimulated an increase in intracellular Ca²⁺ release in DOX(+) cells (Fig. 4N). An additional internal control included absence of effect of 5-HT on Ca²⁺ release in untransfected HEK293 cells (Fig. 4N). This findings indicate that we were able to reproduce the previous observed phenotype

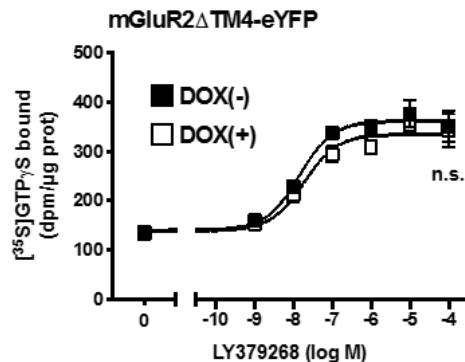


(Fig. 4)

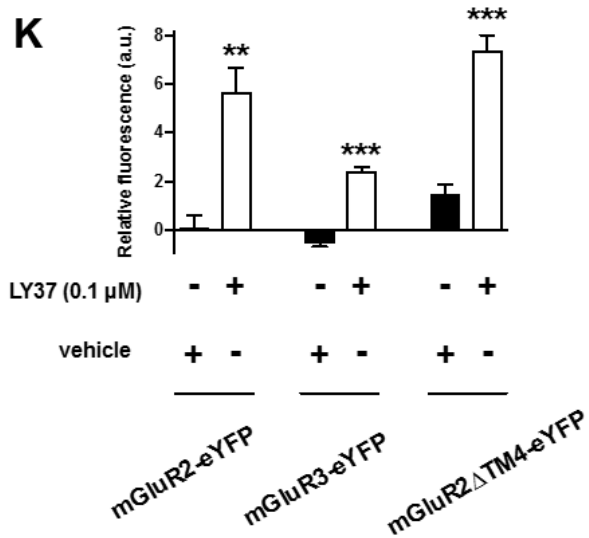
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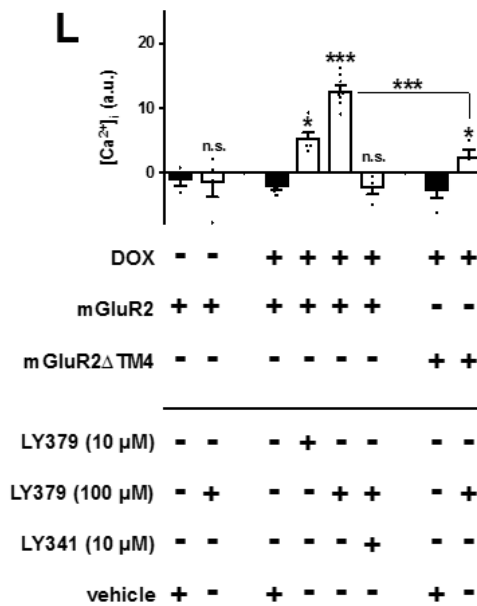
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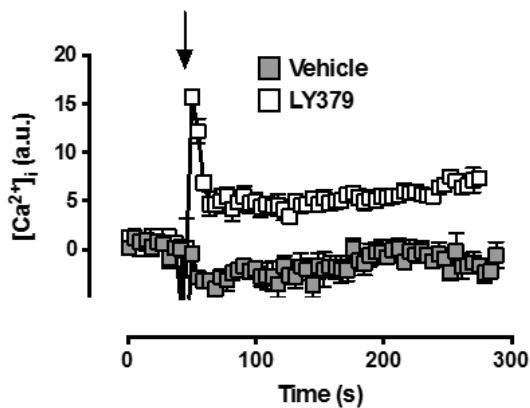
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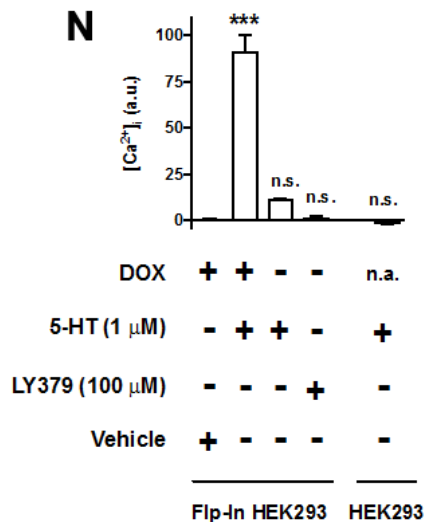
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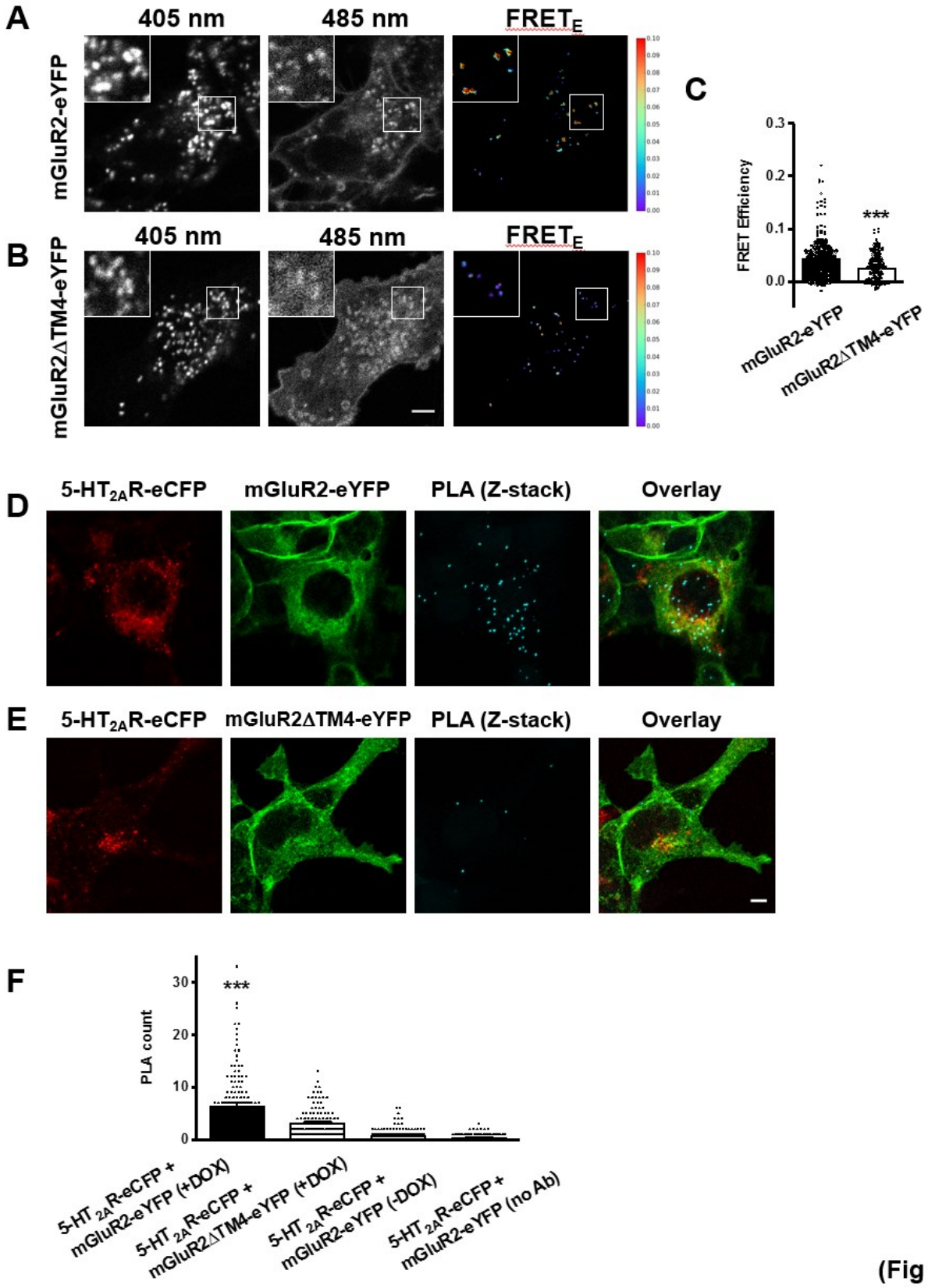
(Fig. 4)

Fig. 4. Characterization of the double stable cells lines. (A to C) Density of 5-HT_{2A}R as measured by [³H]ketanserin binding in cell membrane preparations of Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP (A), mGluR3-eYFP (B), or mGluR2ΔTM4-eYFP (C) and harboring 5-HT_{2A}R-eCFP at the inducible locus. Cells were left untreated [DOX(-)] or treated with doxycycline [DOX(+)] (representative results of three independent experiments performed in duplicate; see also Table 1). (D to F) Density of mGluR2/3 shown as [³H]LY341495 binding in cell membrane preparations of Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP (D), mGluR3-eYFP (E), or mGluR2ΔTM4-eYFP (F) and harboring 5-HT_{2A}R-eCFP at the inducible locus. Cells were untreated [DOX(-)] or treated with doxycycline [DOX(+)] (representative results of three independent experiments performed in duplicate; see also Table 1). (G and H) Cells were untreated [DOX(-)] or treated with doxycycline [DOX(+)], and densities of 5-HT_{2A}R-eCFP and mGluR2-eYFP with eCFP (G) and eYFP (H), respectively, were assessed by FACS assays (n = 10 independent experiments with 8459 – 9710 cells per experimental condition). (I and J) Effect of the mGluR2/3 agonist LY379268 on [³⁵S]GTPγS binding in membrane preparations of Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP (I) or mGluR2ΔTM4-eYFP (J) and harboring 5-HT_{2A}R-eCFP at the inducible locus. (K) [Ca²⁺]_i mobilization of mGluRs clones by activation of the chimeric Gα protein Gα_{qi9}. Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP, mGluR3-eYFP or mGluR2ΔTM4-eYFP and harboring 5-HT_{2A}R-eCFP at the inducible locus were untreated [DOX(-)], loaded with Fura-2 and monitored for intracellular calcium concentration after administration of LY379269 (0.1 μM), or vehicle. To allow efficient coupling of Gα_{i/o}-coupled receptors (mGluR2, mGluR3 and mGluR2ΔTM4) to the PLC pathway, cells were also transfected with the chimeric Gα protein Gα_{qi9}. Analysis of the fold increase in [Ca²⁺]_i (n = 4 independent experiments, representative results of three independent experiments performed in duplicate). (L and M) Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP or mGluR2ΔTM4-eYFP and harboring 5-HT_{2A}R-eCFP at the inducible locus were untreated [DOX(-)] or treated with doxycycline [DOX(+)], loaded with Fura-2 and monitored for intracellular calcium concentration after sequential administration of LY341495 and/or LY379268, or vehicle. Representative time course of Ca²⁺ release. The arrowhead indicates the time when drugs were added (M). Analysis of the fold increase in intracellular calcium concentration (L; n = 4 – 8 independent experiments). (N) [Ca²⁺]_i mobilization of mGluRs clones expressing 5-HT_{2A}R only, or untransfected HEK293 cells. Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP and harboring 5-HT_{2A}R-eCFP at the inducible locus or untransfected HEK293 cells were treated with doxycycline (or left untreated), loaded with Fura-2 and monitored for intracellular calcium concentration after administration of 5-HT (1 μM) or LY379268 (100 μM), or vehicle (n = 4 independent experiments). Data are mean ± SEM (D, I, J, K, L and N). Statistical analysis was performed using the F test (A to F), Student's t-test (G and H) one-way (D, K and N) or two-way (M) ANOVA with Bonferroni's *post hoc* test, or the F test (A to F). *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant.

with our cell system and that the receptors stoichiometry allowing the crosstalk between 5-HT_{2A}R and mGluR2 is conserved²⁵⁴.

V. mGluR2 and 5-HT_{2A}R interact intracellularly

Besides attesting of the coupling of mGluR2 and 5-HT_{2A}R assessed by the above-mentioned BiFC assay, this experiment also showed that the distribution of the fluorescence produced by the complementation of the two mCitrine fluorescent protein moieties held by each receptor is at the cell borders but more importantly, intracellular (Fig. 1G). To further explore where the interaction between these two receptors is localized, we tested RRI using two different methods: sensorFRET, which enables quantitative measurement of FRET efficiency, and proximity ligation assay (PLA), which permits direct detection of molecular interactions between two proteins without the need of resonance energy transfer assays. Using the sensorFRET approach, we observed a significant decrease of FRET efficiency between eCFP and eYFP in DOX(+) cells stably expressing mGluR2 Δ TM4-eYFP as compared to that observed in cells stably expressing mGluR2-eYFP (Figs. 5, A to C and Fig. A1). This confirms the impaired ability of the mGluR2/mGluR3 chimeric construct to physically interact with 5-HT_{2A}R. More importantly, the FRET signal between 5-HT_{2A}R-eCFP and mGluR2-eYFP was located intracellularly in vesicles positive for both eCFP and eYFP fluorescence signal (Fig. 5A). Following DOX-dependent induction of 5-HT_{2A}R-eCFP expression, PLA signal between 5-HT_{2A}R and mGluR2 was observed in the form of punctate staining (Fig. 5D). Quantification of PLA dots was significantly reduced in cells induced to express 5-HT_{2A}R-eCFP along with mGluR2 Δ TM4-eYFP (Figs. 5, E and F). As anticipated, no PLA signal was observed in the absence of doxycycline in cells expressing mGluR2-eYFP only (Fig. 5F). Additional internal controls included omission of one of the primary antibodies in the PLA assays (Fig. 5F).



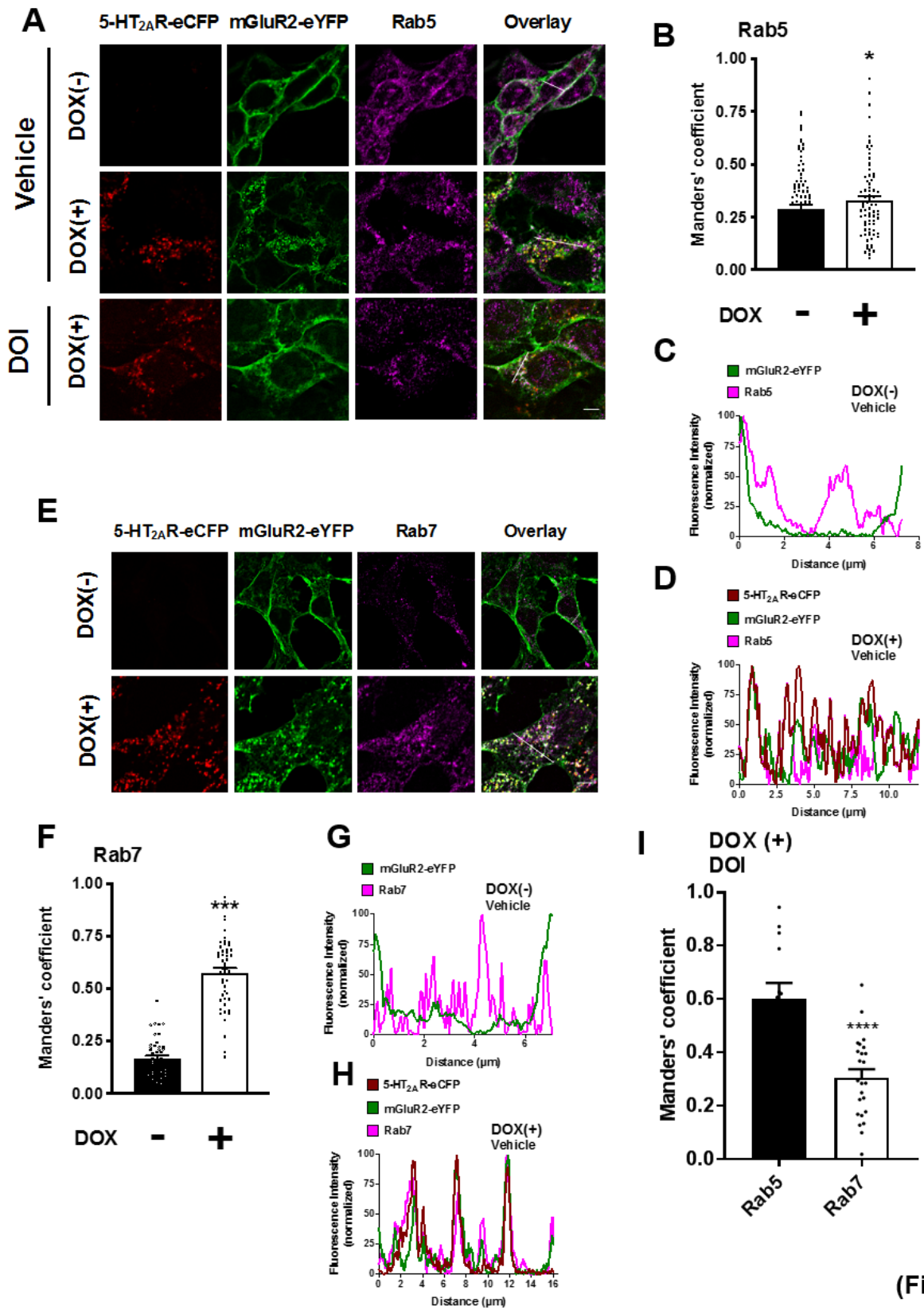
(Fig 5)

Fig. 5. Intracellular localization of the 5-HT_{2A}R-mGluR2 complex. (A to C) SensorFRET analysis of Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP (A and C) or mGluR2 Δ TM4-eYFP (B and C), harboring 5-HT_{2A}R-eCFP at the inducible locus, and treated with doxycycline. Representative live-cell confocal images at 405 nm and 458 nm excitation frequencies (A and B), and (C) quantification of FRET efficiencies (n = 249 – 364 regions of interest in three independent experiments). (D to F) Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP (D and F) or mGluR2 Δ TM4-eYFP (E and F) and harboring c-Myc-5-HT_{2A}R-eCFP at the inducible locus were treated with doxycycline, permeabilized and stained with anti-rabbit antibody selective for c-Myc and anti-mouse antibody selective for mGluR2, followed by incubation with species-specific PLA probes. Representative confocal micrographs of eCFP- or eYFP-tagged constructs and PLA signal (cyan dots, Z-stack projection) (D and E). Quantification of PLA dots (F). Note that PLA signal was decreased in DOX(-) cells, and when primary antibodies were not added (n = 57 – 94 cell regions of interest demarcated according to eYFP signal within previously defined eCFP-positive cells in three independent experiments). Data are median with 95% confidence interval (C) or mean \pm SEM (F). Statistical analysis was performed using Mann-Whitney U test (C) or one-way ANOVA with Bonferroni's *post hoc* test (F). ****P* < 0.001. Scale bars, 5 μ m, inserts edge \sim 14.3 μ m.

VI. Expression of 5-HT_{2A}R augments localization of mGluR2 in endosomal compartments

Our data thus far show that, under steady state conditions, colocalization of mGluR2 with markers of endocytic vesicles increased upon coexpression with 5-HT_{2A}R. However, the nature of these intracellular compartments remains unknown. It is classically established that agonist binding and activation of most 7TMRs usually results in the rapid removal of the receptor from the plasma membrane by two complementary mechanisms, desensitization and endocytosis. To evaluate the components of the intracellular trafficking pathway in which 5-HT_{2A}R affects subcellular distribution of mGluR2, we next tested the effect of DOX-induced expression of 5-HT_{2A}R on colocalization of mGluR2 with endosomal markers. Using fixed cell confocal immunofluorescence microscopy, we found that colocalization of mGluR2-eYFP with Rab5, which regulates clathrin-mediated endocytosis from the plasma membrane to early/sorting endosome pools, showed a modest, yet statistically significant, increase in DOX(+) cells as compared to DOX(-) cells (Figs. 6, A to D). Additionally, DOX-induced expression of 5-HT_{2A}R substantially augmented localization of mGluR2-eYFP with Rab7 as a late endosome maker (Figs. 6, E to H).

This localization of mGluR2 in Rab7 endosomes was surprising. Are those receptors directed to degradation, or Rab7 endosomes serve as storage compartments that are deeper or later used after endocytosis? To address that question, colocalization of mGluR2 in Rab7 endosomes was assessed following activation of the 5-HT_{2A} receptor. Application of DOI resulted in the reversal of the previous pattern; mGluR2 population was now greater in Rab5 than it was in Rab7 endosomes (Fig. 6I). This observation suggests that following activation of 5-HT_{2A}R, a Rab transition demobilized the Rab5 pool of mGluR2 to Rab7. It is possible that 5-HT_{2A}R activation leads to the migration of mGluR2 from Rab7 to Rab5 endosomes. This could explain why at basal state, *i.e.* in absence of 5-HT_{2A}R and mGluR2 ligand, the conservation of mGluR2 is maintained in late endosomes.



(Fig 6)

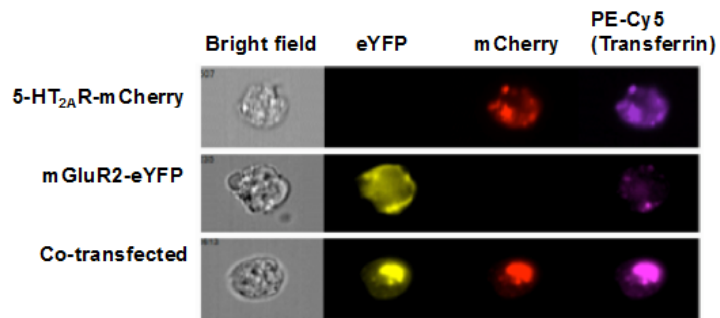
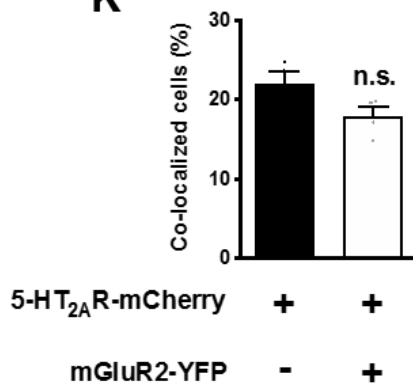
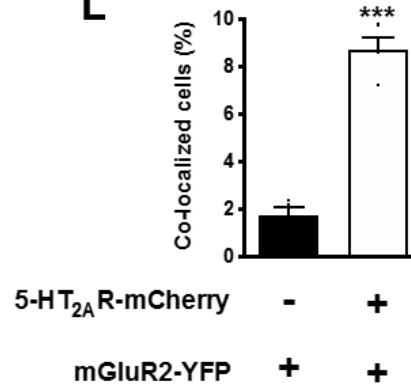
J**K****L****(Fig 6)**

Fig. 6. Agonist activation of either 5-HT_{2A}R or mGluR2 differentially affects mGluR2 trafficking and downregulation. (A to L) Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP and harboring c-Myc-5-HT_{2A}R-eCFP at the inducible locus were untreated [DOX(-)] or treated with doxycycline [DOX(+)], and exposed for 60 min to DOI (1 μ M). Cells were then permeabilized and stained with anti-Rab5 or anti-Rab7 and secondary antibody, and imaged by confocal microscopy to detect eCFP, eYFP, anti-Rab5, or anti-Rab7. Representative confocal micrographs (A and E) and corresponding line scans (C and D, G and H), and Manders' colocalization coefficient analysis of anti-Rab5 (A and B; n = 33 – 134 cell regions of interest in three independent experiments) or anti-Rab7 (E and F; n = 48 – 49 cell regions of interest in three independent experiments) and eYFP-tagged construct. (I) Manders' colocalization coefficient analysis between Rab5 and Rab7 in [DOX(+)] cells and treated with DOI (1 μ M) (36 cell regions of interest in two independent experiments). (J to L) FACS analysis of internalized mGlu2 receptors along with the transferrin marker. (J and L) HEK293 cells were transfected to express 5-HT_{2A}R-mCherry alone, mGluR2-eYFP alone, or 5-HT_{2A}R-mCherry and mGluR2-eYFP together, permeabilized, stained with PE-Cy5-tagged anti-transferrin receptor antibody, and imaged with an Amnis ImageStream flow cytometer. (J) Representative images. (K) Colocalization of 5-HT_{2A}R-mCherry and transferrin receptor with or without coexpression of mGluR2-eYFP (n = 4 independent experiments). (L) Colocalization of mGluR2-eYFP and transferrin receptor with or without coexpression of 5-HT_{2A}R-mCherry (n = 4 independent experiments). Data are mean \pm SEM (B, F, I, K and L). Statistical analysis was performed using the Student's *t*-test (B, F, I, K and L). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, n.s., not significant. Scale bars, 5 μ m.

To pursue quantitative comparison of variant receptor trafficking, and to validate our previous findings with an independent marker of clathrin-mediated endosomes, we assessed colocalization of 5-HT_{2A}R-mCherry or mGluR2-eYFP with the transferrin receptor, which transits through clathrin-coated pits and behaves as a marker of early endosomes, using imaging flow cytometry. As above with Rab5 and Rab7, our data show that localization between 5-HT_{2A}R-mCherry and the transferrin receptor was higher as compared to that observed between mGluR2-eYFP and transferrin receptor (Fig. 6J). Additionally, we show that whereas colocalization between 5-HT_{2A}R-mCherry and transferrin receptor was not affected upon cotransfection of the mGluR2-eYFP construct (Fig. 6K), transfection of the 5-HT_{2A}R-mCherry construct led to a significant increase in colocalization between mGluR2-eYFP and the transferrin receptor (Fig. 6L).

VII. DOI and LY379268 differentially affect localization of mGluR2 in Rab5-positive endosomes

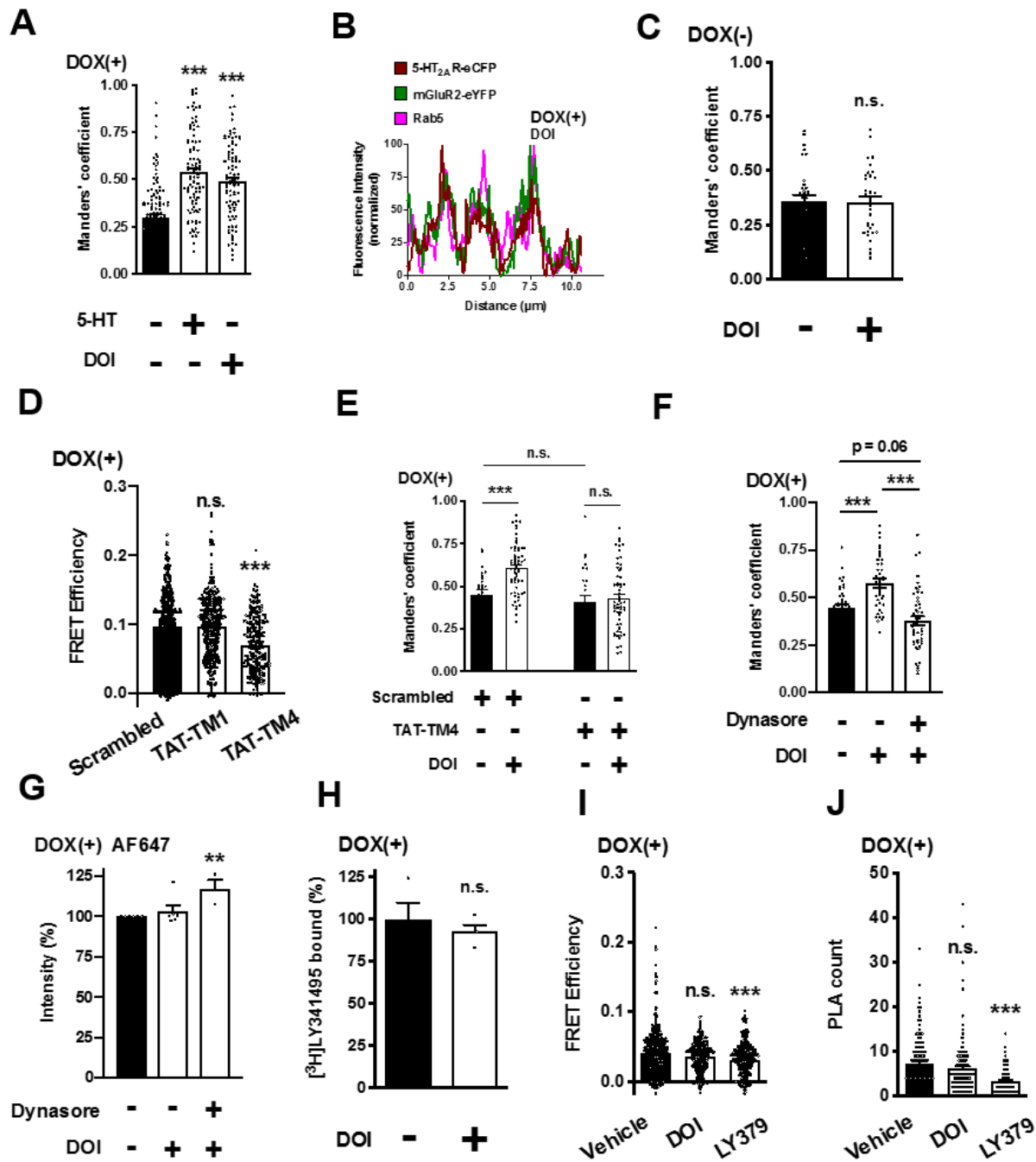
To gain further insight into how cells control the subcellular localization of a 7TMR complexes, we next investigated the effects of different serotonergic and glutamatergic agonists on trafficking processes in cells coexpressing 5-HT_{2A}R and mGluR2.

We first explored the effect of the endogenous neurotransmitter serotonin (5-HT) or the 5-HT_{2A}R agonist DOI on localization of mGluR2 within Rab5-positive endosomes. Our data showed that colocalization of mGluR2-eYFP and Rab5 was significantly increased after either 5-HT or DOI treatment in cells stably expressing mGluR2-eYFP and induced to express 5-HT_{2A}R-eCFP (Figs. 6A, and 7A, and B). To rule out any effect of DOI on mGluR2 trafficking, we confirmed that treatment with DOI does not affect localization of mGluR2-eYFP and Rab5 in cells stably expressing mGluR2-eYFP and uninduced (DOX[-]) to express 5-HT_{2A}R-eCFP (Fig. 7C).

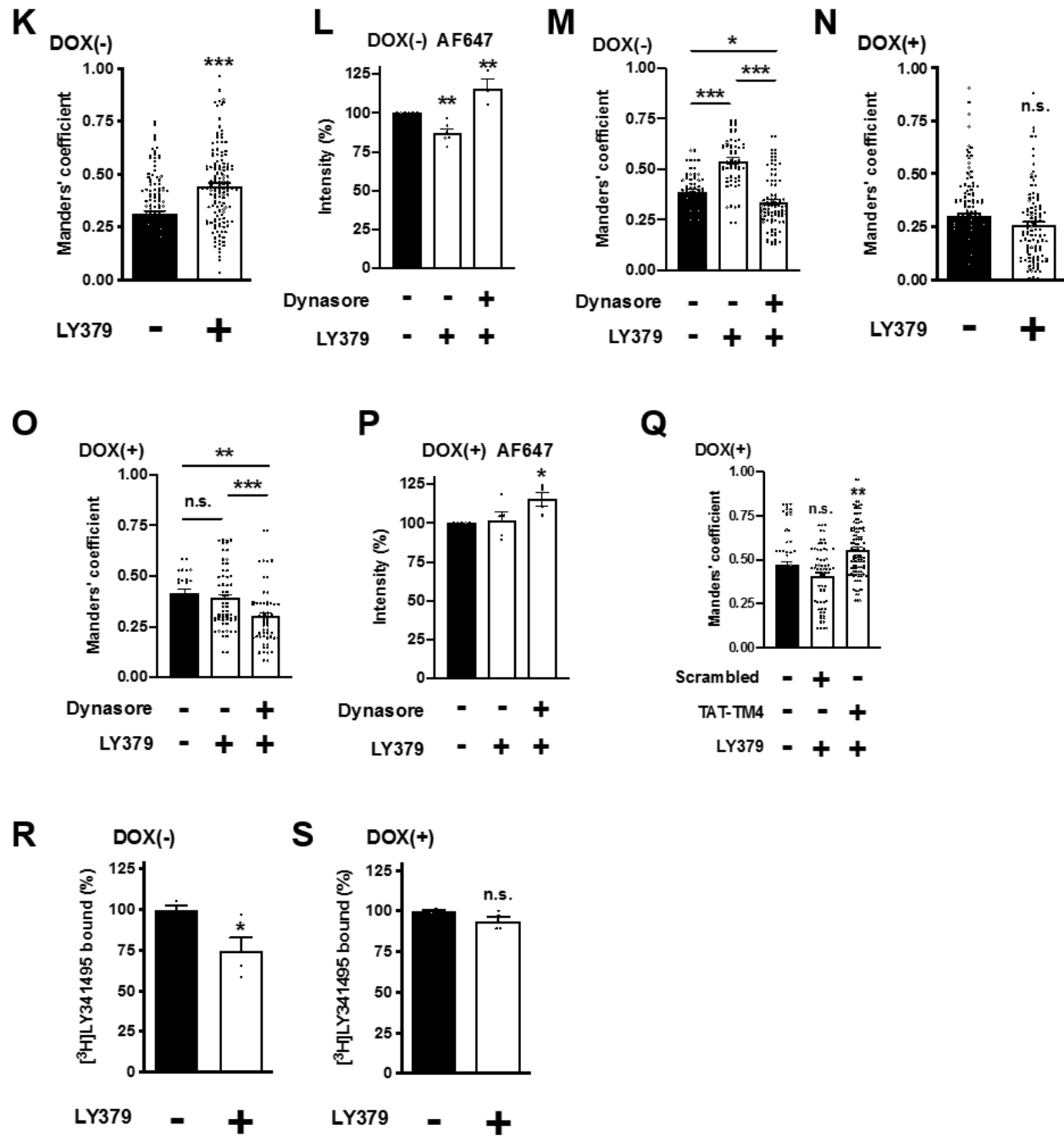
To test whether 5-HT_{2A}R and mGluR2 receptor heteromer is required for this trafficking event, we used synthetic peptides with the amino acid sequence of TM4 of mGluR2 fused to the HIV

transactivator of transcription (TAT) peptide, which determines the orientation of the peptide when inserted in the plasma membrane. To begin with, we wanted to test whether the interfering peptide can reduce or even prevent the interaction between 5-HT_{2A} and mGluR2. As expected and based on our above findings, incubation with TAT-TM4, but not with TAT-TM1 or TAT-tagged scrambled peptide (TAT-SCR), significantly decreased FRET signal in cells coexpressing 5-HT_{2A}R-eCFP and mGluR2-eYFP (Fig. 7D). Notably, incubation with TAT-TM4 reduced the effect of DOI on colocalization of mGluR2-eYFP and Rab5 (Fig. 7E). Exposure to TAT-TM4, however, did not affect colocalization between mGluR2-eYFP and Rab5 in vehicle-treated cells (Fig. 7E). To gain further insight into the endocytic process at work, endocytosis was pharmacologically blocked by an inhibitor. Dynamin is a GTPase protein that is essential for membrane fission during clathrin-mediated endocytosis. Dynasore acts as a potent inhibitor of endocytic pathways known to depend on dynamin by rapidly blocking coated vesicle formation within seconds of its application. Cells coexpressing 5-HT_{2A}R and mGluR2 was submit to dynasore mediated endocytosis inhibition and treated with DOI. As a result, a diminution of the effect of DOI on augmentation of colocalization between mGluR2-eYFP and Rab5 was observed (Fig. 7F).

To validate some of above findings with a different approach, and to track mGluR2 internalization upon different treatments, we processed the cells to flow cytometry, where cell surface localization of mGluR2-eYFP is detected thanks to an Alexa Fluor 647 (AF647)-tagged antibody targeting an epitope of the N-terminal of mGluR2. Surprisingly, mGluR2 surface distribution was unaffected by DOI in DOX(+) cells (Fig. 7G). As expected, however, cell surface AF647 signal was augmented upon dynasore treatment (Fig. 7G). This lack of effect of DOI exposure on cell surface mGluR2-eYFP was further supported by radioligand binding assays. [³H]LY341495 binding was comparable in DOX(+) cells treated with DOI and vehicle (Fig. 7H). Together with the absence of effect of DOI treatment on FRET (Fig. 7I) and PLA (Fig. 7J) signal between 5-HT_{2A}R-eCFP and mGluR2-eYFP, these data suggest that whereas exposure to DOI augments subcellular



(Fig 7)



(Fig 7)

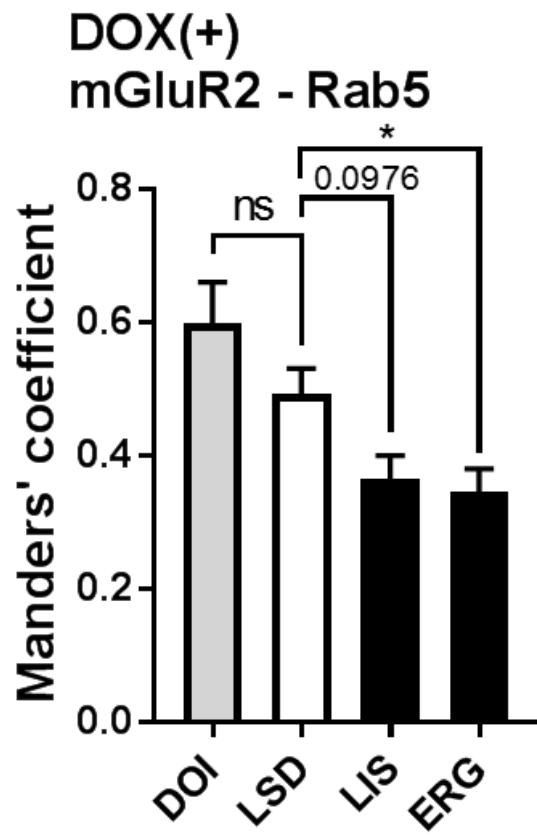
Fig. 7. DOI and LY379268 differentially affect localization of mGluR2 in Rab5-positive endosomes. (A to C, K and N) Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP and harboring c-Myc-5-HT_{2A}R-eCFP at the inducible locus were untreated [DOX(-)] or treated with doxycycline [DOX(+)], and exposed for 60 min to serotonin (5-HT, 1 μ M), DOI (1 μ M) or LY379268 (10 μ M), or vehicle. Cells were then permeabilized and stained with anti-Rab5 and secondary antibody, and imaged by confocal microscopy to detect eCFP, eYFP, anti-Rab5 (Fig. 6A 3rd line). Representative line scan of DOI treated cells (B). Cells were incubated (60 min; 10 μ M) with TAT-fused peptide corresponding to TM1 (TAT-TM1) or TM4 (TAT-TM4) of mGluR2, or scrambled control peptide and then processed for sensorFRET (n = 248 – 393 regions of interest in three independent experiments) (D). (E and Q) Manders' coefficient colocalization analysis of anti-Rab5 and eYFP-tagged construct. Cells were exposed for 60 min to DOI (1 μ M, E), LY379268 (10 μ M, Q), or vehicle. TAT-tagged peptides (10 μ M) were added 5 min before drug or vehicle administration (n = 27 – 103 cell regions of interest in three independent experiments). (F, M and O) Manders' coefficient colocalization analysis of anti-Rab5 and eYFP-tagged construct. Cells were exposed for 60 min to DOI (1 μ M, F), LY379268 (10 μ M, M and O), or vehicle. Dynasore (80 μ M) was added 5 min before drug or vehicle administration (n = 28 – 88 cell regions of interest in three independent experiments). (G, L and P) Cell surface localization of mGluR2-eYFP assessed by flow cytometry assays with an Alexa Fluor 647 (AF647)-tagged antibody. Cells were exposed for 60 min to DOI (1 μ M, G), LY379268 (10 μ M, L and P), or vehicle. Dynasore (80 μ M) was added 5 min before drug or vehicle administration (n = 3 – 6 independent experiments with 8508 – 10048 cells per experimental condition). (H, R and S) Density of mGluR2 shown as [³H]LY341495 binding in membrane preparations of DOX(+) (H and S) or DOX(-) (R) cells exposed for 60 min to DOI (1 μ M, H), LY379268 (10 μ M, R and S), or vehicle. Data are shown as percentage of specific binding in DOI- or LY379268-treated cells as compared to vehicle (n = 4 independent groups of membrane preparations). (I and J) Cells were exposed for 60 min to DOI (1 μ M), LY379268 (10 μ M), or vehicle. Quantification of FRET efficiencies (I, n = 281 – 536 regions of interest in three independent experiments). Quantification of PLA dots (J, n = 82 – 148 cell regions of interest demarcated according to eYFP signal within previously defined eCFP-positive cells in three independent experiments). Data are mean \pm SEM (A, C, E to H, K to S) or median with 95% confidence interval (D, I and J). Statistical analysis was performed using Student's *t*-test (C, H, K, N, R, S), one-way ANOVA with Bonferroni's *post hoc* test (A, F, G, L, M, O, and P and Q), two-way ANOVA with Bonferroni's *post hoc* test (E), or one-way non-parametric ANOVA (Kruskal-Wallis) with Dunn's *post hoc* test (D, I and J). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s., not significant.

localization of mGluR2 within Rab5-positive endosomes through a mechanism that requires heteromerization between 5-HT_{2A}R and mGluR2, activation of the 5-HT_{2A}R receptor does not affect mGluR2 cell surface density, total density or heteromerization with 5-HT_{2A}R.

We next tested whether DOX-induced expression of 5-HT_{2A}R-eCFP modulates the effect of the mGluR2/3 agonist LY379268 on mGluR2's subcellular localization and trafficking. Consistent with the paradigmatic description of 7TMR endocytosis after ligand binding, in DOX(-) cells, incubation with LY379268 led to both an increase of colocalization between mGluR2-eYFP and Rab5 (Fig. 7K), and reduced cell surface AF647 signal (Fig. 7L), phenotypes that were prevented by dynasore (Figs. 7, L and M). Importantly, this effect of LY379268 on subcellular localization of mGluR2 was not observed in DOX(+) cells (Figs. 7, N, O and P), although dynasore augmented the surface location of mGluR2. This effect was rescued in DOX(+) cells treated with TAT-TM4 (Fig. 7Q). Additionally, our radioligand bindings assays with the mGluR2/3 antagonist [³H]LY341495 showed that mGluR2 density was reduced in DOX(-) cells treated with LY379268 (Fig. 7R), whereas LY379268 treatment did not affect [³H]LY341495 binding in cells stably expressing mGluR2-eYFP and induced to express 5-HT_{2A}R-eCFP (Fig. 7S). By contrast, we observed that, in DOX-treated cells, molecular proximity between 5-HT_{2A}R-eCFP and mGluR2-eYFP, as assessed by sensorFRET (Fig. 7I) and PLA (Fig. 7J), was reduced upon previous exposure to LY379268. Together, these data indicate that the 5-HT_{2A}R agonist DOI and the mGluR2/3 agonist LY379268 differentially affect mGluR2 density as well as localization of mGluR2 with markers of endocytic compartments in cells expressing mGluR2 alone, or together with 5-HT_{2A}R.

VIII. Psychedelic and non-psychedelic compounds have different effects on the 5-HT_{2A}R-mediated endocytosis of mGluR2

Our system thus far showed that 5-HT_{2A}R agonists such as DOI and 5-HT displaced mGluR2 to different marked compartment of the endocytic system, and that is dependent on the 5-HT_{2A}-



(Fig 8)

Fig. 8. Activation of 5-HT_{2A}R by hallucinogenic or nonhallucinogenic agonists differently mediate Rab5-dependent trafficking of mGluR2. Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP and harboring c-Myc-5-HT_{2A}R-eCFP at the inducible locus were treated with doxycycline [DOX(+)], and exposed for 60 min to DOI (1 μM), LSD (1μM), lisuride (1μM) or ergotamine (1μM). Cells were then permeabilized and stained with anti-Rab5 and secondary antibody, and imaged by confocal microscopy to detect eCFP, eYFP, anti-Rab5. Manders' coefficient colocalization analysis of anti-Rab5 (n = 33 cell regions of interest in one experiment) and eYFP-tagged construct. Data are mean ± SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni's post hoc test. *P < 0.05, n.s., not significant.

mGluR2 receptor heterocomplex. Although serotonin is not hallucinogenic at physiological concentration, unlike DOI, we wanted to gain more insights into how propsychotic molecules such as 5-HT_{2A}R hallucinogens (HAL) can affect mGluR2 trafficking as compared to non-hallucinogenic agents (NHAL). Hallucinogenic 5-HT_{2A}R agonists such as LSD and psilocybin have been categorized as propsychotic compounds because of their ability to mimic some of the schizophrenia positive symptoms. Conversely, molecules that are very close in structure but not hallucinogenic, such as lisuride and ergotamine, seem to evoke different internalization and signaling mechanism, such as bias agonism^{114,352} and transphosphorylation³⁵⁰. Given that HAL and NHAL have different effects on the internalization of 5-HT_{2A}R and that it can phosphorylate mGluR2 to affect its Gai/o signaling and probably its internalization following exposure to the drugs, we hypothesized that similar to those findings, HAL and NHAL would have different effects on the internalization of mGluR2 in Rab5 endosomes in cells coexpressing both receptors. Consistently with previous findings^{163,350}, and similar to the findings with 5-HT and DOI, cells treated with LSD and psilocybin tended to have a greater colocalization index between mGluR2 and Rab5 (Fig. 8) (Fig. 8). Conversely, no increase was observed with NHAL treatments such as lisuride and ergotamine, indicating that only 5-HT_{2A}R hallucinogenic agonists have the potency to affect mGluR2 trafficking (Fig. 8).

IX. Exposure to clozapine down-regulates mGluR2 via GPCR heteromerization with

5-HT_{2A}R

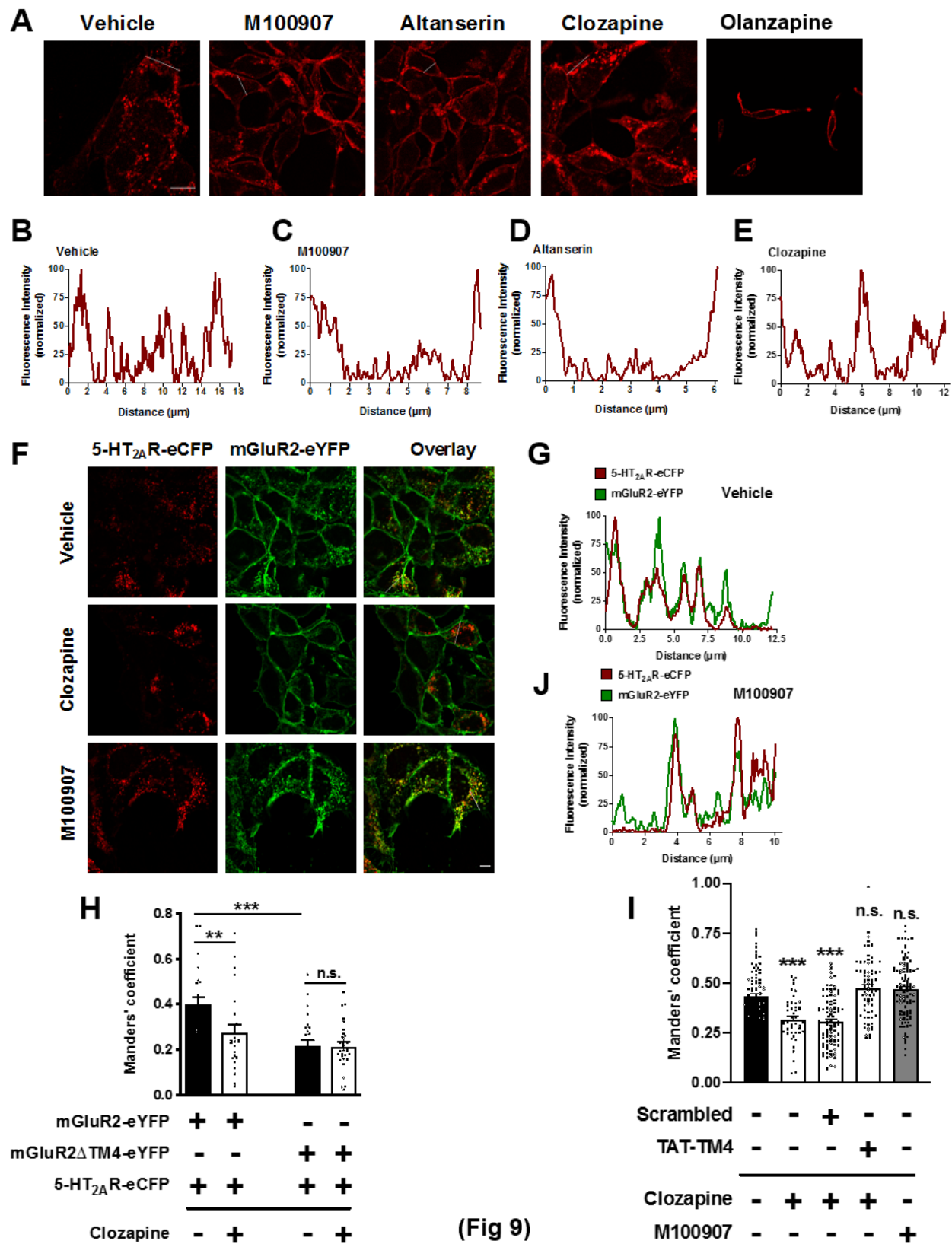
1. Effect of 5-HT_{2A}R antagonists on receptor's subcellular distribution

Recent reports suggest that presence of 5-HT_{2A}R antagonists/inverse agonists affects intracellular localization of 5-HT_{2A}R constructs in living mammalian cell cultures. As an example, upon addition of ligands such as mianserin, cells show 5-HT_{2A}Rs predominantly at the plasma membrane³³⁵. To corroborate these findings, we used our Flp-In T-REx system expressing 5-HT_{2A}R-eCFP only (*i.e.*, without stable expression of mGluR2-eYFP, mGluR3-eYFP or mGluR2ΔTM4) in an inducible

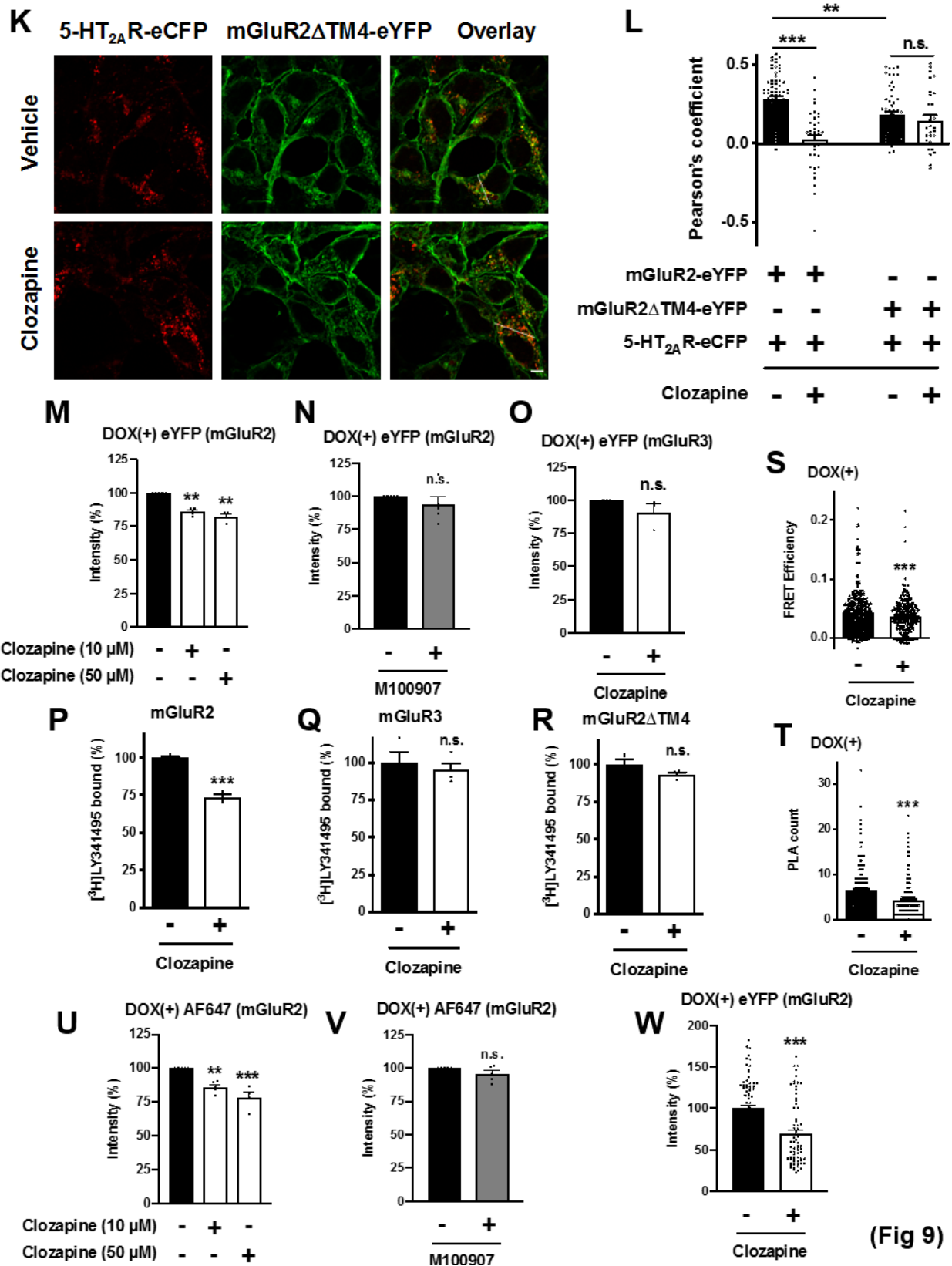
manner. Following addition of DOX, cells were treated overnight with the 5-HT_{2A}R antagonist/inverse agonist M100907 (also known as volinanserin or MDL 100,907), or vehicle. Our data indicated that, opposite to that observed in vehicle-treated cells, 5-HT_{2A}R-eCFPs were essentially localized at the plasma membrane of cells treated with M100907 (Fig. 9A to C). Similar results were obtained with the 5-HT_{2A}R antagonist/inverse agonist altanserin (Fig. 9A and D). Surprisingly, the clozapine's analog olanzapine had the same effect on the localization of 5-HT_{2A}R (Fig. 9A). Together, these data suggest that, at steady state, the bulk of 5-HT_{2A}R-eCFP is present in punctate intracellular vesicles, and that treatment with the 5-HT_{2A}R antagonists/inverse agonists enhances the otherwise restricted cell surface localization of the 5-HT_{2A}R.

2. Effect of clozapine on 5-HT_{2A}R's subcellular distribution

Clozapine, however, is an antipsychotic medication that presents peculiar properties as it normally behaves as a 5-HT_{2A}R antagonist/inverse agonist, thus blocking or inhibiting 5-HT_{2A}R-dependent principal signaling pathways such as activation of G $\alpha_{q/11}$ proteins, yet is also able to activate certain pathways such as Akt. Additionally, and opposite to what is usually observed with most antagonists and inverse agonists (see above), our data showed that following DOX-induced expression of 5-HT_{2A}R-eCFP, clozapine exposure did not discernibly affect the subcellular distribution of the 5-HT_{2A}R construct (Fig. 9A and E). Nevertheless, our previous findings in whole animal models showed that long-lasting exposure to clozapine induces mGluR2 down-regulation in brain regions such as the frontal cortex. Consequently, we tested whether clozapine affects mGluR2 localization and density in cells coexpressing 5-HT_{2A}R and mGluR2, as well as the extent to which 5-HT_{2A}R-mGluR2 heteromerization is necessary for this trafficking event.



(Fig 9)



(Fig 9)

Fig. 9. Clozapine treatment affects mGluR2 trafficking and downregulation via 5-HT_{2A}R-mGluR2. (A) Representative confocal micrographs and representative line scans of Flp-In T-REx HEK293 cells harboring 5-HT_{2A}R-eCFP at the inducible locus were treated with doxycycline, and then exposed overnight to M100907 (C) (10 μM), altanserin (D) (10 μM) or clozapine (E) (10 μM), olanzapine (10 μM) or vehicle (B). (F to H and K, J) Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP (F, G to L, N, P, and U, V, W), mGluR2ΔTM4-eYFP (H, L, R), or mGluR3-eYFP (O, Q) and harboring 5-HT_{2A}R-eCFP at the inducible locus were treated with doxycycline and then exposed overnight to clozapine (10 μM or 50 μM) or M100907 (10 μM), or vehicle. Representative confocal micrographs and line scans (F, G, J, K). (H) Manders' coefficient colocalization analysis of eYFP- and eCFP-tagged constructs (n = 30 – 74 cell regions of interest in three independent experiments). (I) Manders' coefficient colocalization analysis of eYFP- and eCFP-tagged constructs in cells stably expressing mGluR2-eYFP and harboring 5-HT_{2A}R-eCFP at the inducible locus. Cells were treated with doxycycline, and then exposed overnight to clozapine (10 μM) or M100907 (10 μM), or vehicle. TAT-tagged peptides were added both 5 minutes before clozapine or vehicle administration and 65 minutes before cell fixation (n = 44 – 98 cell regions of interest in three independent experiments). (M to O, U, V, W) Cell surface localization of mGluR2-eYFP with an Alexa Fluor 647 (AF647)-tagged antibody (U, V, W) and mGluR2-eYFP (M, N) or mGluR3-eYFP (O) density with eYFP were assessed by flow cytometry assays (n = 3 – 5 independent experiments with 8148 – 10418 cells per experimental condition). (P to R) Density of mGluR2 (P), mGluR3 (Q) or mGluR2ΔTM4 (R) shown as [³H]LY341495 binding in membrane preparations of cells exposed overnight to clozapine (10 μM), or vehicle (n = 4 independent groups of membrane preparations). (S) Quantification of FRET efficiencies (n = 315 – 364 regions of interest in three independent experiments). (T) Quantification of PLA dots (n = 160 – 253 cell regions of interest demarcated according to eYFP signal within previously defined eCFP-positive cells in three independent experiments). (W) Intracellular eYFP signal in cells stably expressing mGluR2-eYFP and harboring 5-HT_{2A}R-eCFP at the inducible locus. Cells were treated with doxycycline, and then exposed overnight to clozapine (10 μM), or vehicle (n = 73 – 68 cell regions of interest demarcated based on eCFP signal within intracellular vesicles in three independent experiments). Data are mean ± SEM (H, and I, L to R, and T to W) or median with 95% confidence interval (S). Statistical analysis was performed using Student's *t*-test (N to R, S, T, and W), one-way ANOVA with Bonferroni's *post hoc* test (M and U), two-way ANOVA with Bonferroni's *post hoc* test (H, I, L), or Mann-Whitney U test (S). ***P* < 0.01, ****P* < 0.001, n.s., not significant. Scale bars, 5 μm.

3. Effect of clozapine on mGluR2's subcellular distribution

Cells induced to express 5-HT_{2A}R-eCFP and stably expressing mGluR2-eYFP were incubated with clozapine or vehicle. As before (see Fig. 3A, above), induction of 5-HT_{2A}R-eCFP corroborated a high level of colocalization between 5-HT_{2A}R-eCFP and mGluR2-eYFP in intracellular vesicles of vehicle-treated cells (Fig. 9F, G and L). Importantly, addition of clozapine resulted in an observable reduction of colocalization between mGluR2-eYFP and 5-HT_{2A}R-eCFP (Figs. 9, F and Hand L). This effect was not observed after addition of M100907 (Figs. 9, F and I), and was prevented by TAT-TM4 (Fig. 9I). Interestingly, and opposite to the effect observed in cells expressing 5-HT_{2A}R-eCFP alone (see Fig. 9A, above), both 5-HT_{2A}R-eCFP and mGluR2-eYFP remained intracellularly colocalized upon exposure to M100907 (Fig. 9F and J). As expected (see Fig. 3C, above), colocalization was less observable in cells coexpressing 5-HT_{2A}R-eCFP and mGluR2 Δ TM4-eYFP (Figs. 9H, K, L), yet this low degree of colocalization was unaffected upon clozapine exposure (Figs. 9F, H and L)). Together, these data suggest that clozapine, and not M100907, reduces subcellular colocalization of 5-HT_{2A}R-eCFP and mGluR2-eYFP through a mechanism that requires their molecular proximity.

4. Effect of clozapine on mGluR2 density and expression and its heteromeric association with 5-HT_{2A}R

We next tested whether clozapine treatment affects mGluR2's density via the 5-HT_{2A}R-mGluR2 heteromer. Our flow cytometry analysis shows that, in DOX(+) cells stably expressing mGluR2-eYFP, clozapine treatment reduced intensity of eYFP (Fig. 9F), an effect that was not observed upon M100907 treatment (Fig. 9N) or in cells stably expressing mGluR3-eYFP (Fig. 9O). Similarly, density of mGluR2-eYFP, as determined by binding saturation curves with [³H]LY341495, was reduced upon clozapine treatment in cells induced to express 5-HT_{2A}R-eCFP and stably expressing mGluR2-eYFP (Fig. 9P and Fig. A2 A). This clozapine-dependent effect, however, was not observed in cells induced to express 5-HT_{2A}R-eCFP and stably expressing either mGluR3-

eYFP (Fig. 9Q and Fig. A2 B) or mGluR2 Δ TM4-eYFP (Fig. 9R and Fig. A2 C). Treatment with clozapine also diminished both FRET efficiency (Fig. 9S) and PLA signal (Fig. 9T) between 5-HT_{2A}R-eCFP and mGluR2-eYFP. Most importantly, although clozapine (Fig. 9U), but not M100907 (Fig. 9V), decreased cell surface immunoreactivity of mGluR2-eYFP, this effect was more evident on the subpopulation of mGluR2 located intracellularly (Fig. 9W). Additional controls included absence of effect of clozapine on cell surface mGluR2-eYFP immunoreactivity (Fig. A2 D5Q) and eYFP signal (Fig. A2 E) in DOX(-) cells.

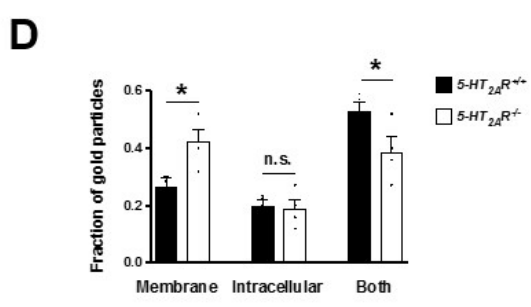
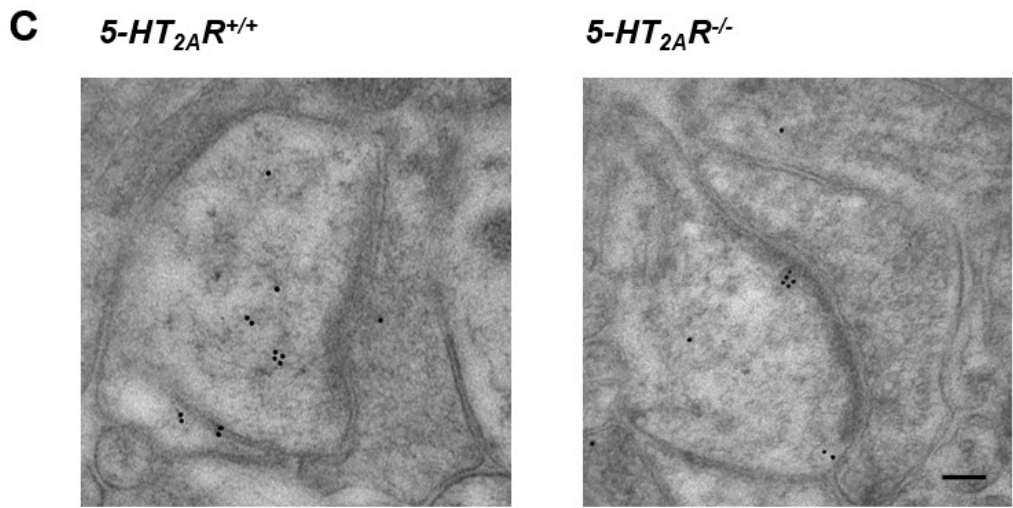
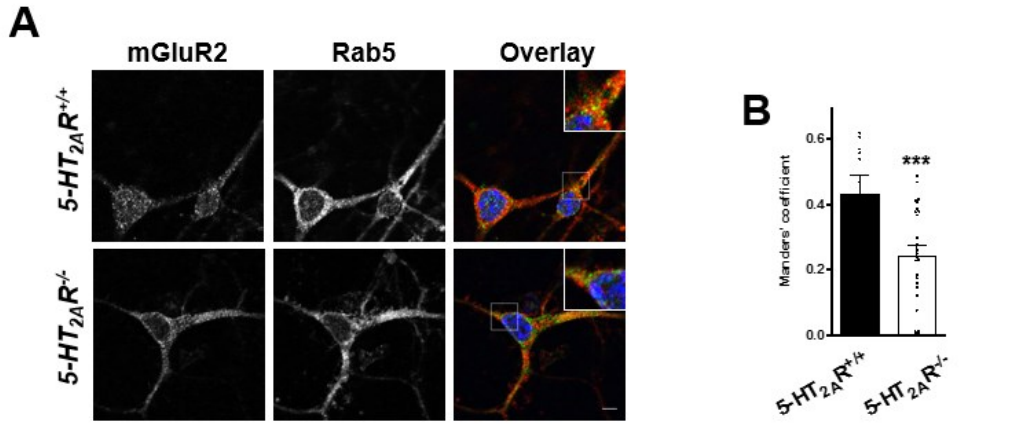
X. Absence of 5-HT_{2A}R expression affects mGluR2 localization in cortical pyramidal neurons

1. Colocalization of mGluR2 and Rab5 in mouse cortical primary neurons

Previous neuroanatomical studies showed that 5-HT_{2A}R and mGluR2 colocalize in frontal cortex pyramidal neurons. Our current data so far suggest that expression of 5-HT_{2A}R modulates mGluR2 trafficking and localization in a mammalian tissue culture model. To ensure that this phenomenon occurs in a primary cell culture system, we examined whether localization of mGluR2 in Rab5-positive endosomes is affected in cortical neuron cultures of 5-HT_{2A}R knockout (*5-HT_{2A}R^{-/-}*) mice and wild-type (*5-HT_{2A}R^{+/+}*) littermates. Notably, colocalization of anti-mGluR2 immunoreactivity and Rab5 was significantly reduced in cortical primary neuron cultures of *5-HT_{2A}R^{-/-}* mice, as compared to *5-HT_{2A}R^{+/+}* animals (Figs. 10, A and B).

2. Effect of 5-HT_{2A}R expression on mGluR2 subcellular localization in mouse frontal cortex synapses

To assess whether the subcellular localization of mGluR2 is regulated by its interactions with 5-HT_{2A}R in native tissue, we examined the distribution of anti-mGluR2 immunoreactivity relative to the plasmalemma in the frontal cortex of *5-HT_{2A}R^{+/+}* and *5-HT_{2A}R^{-/-}* mice using immunogold



(Fig 10)

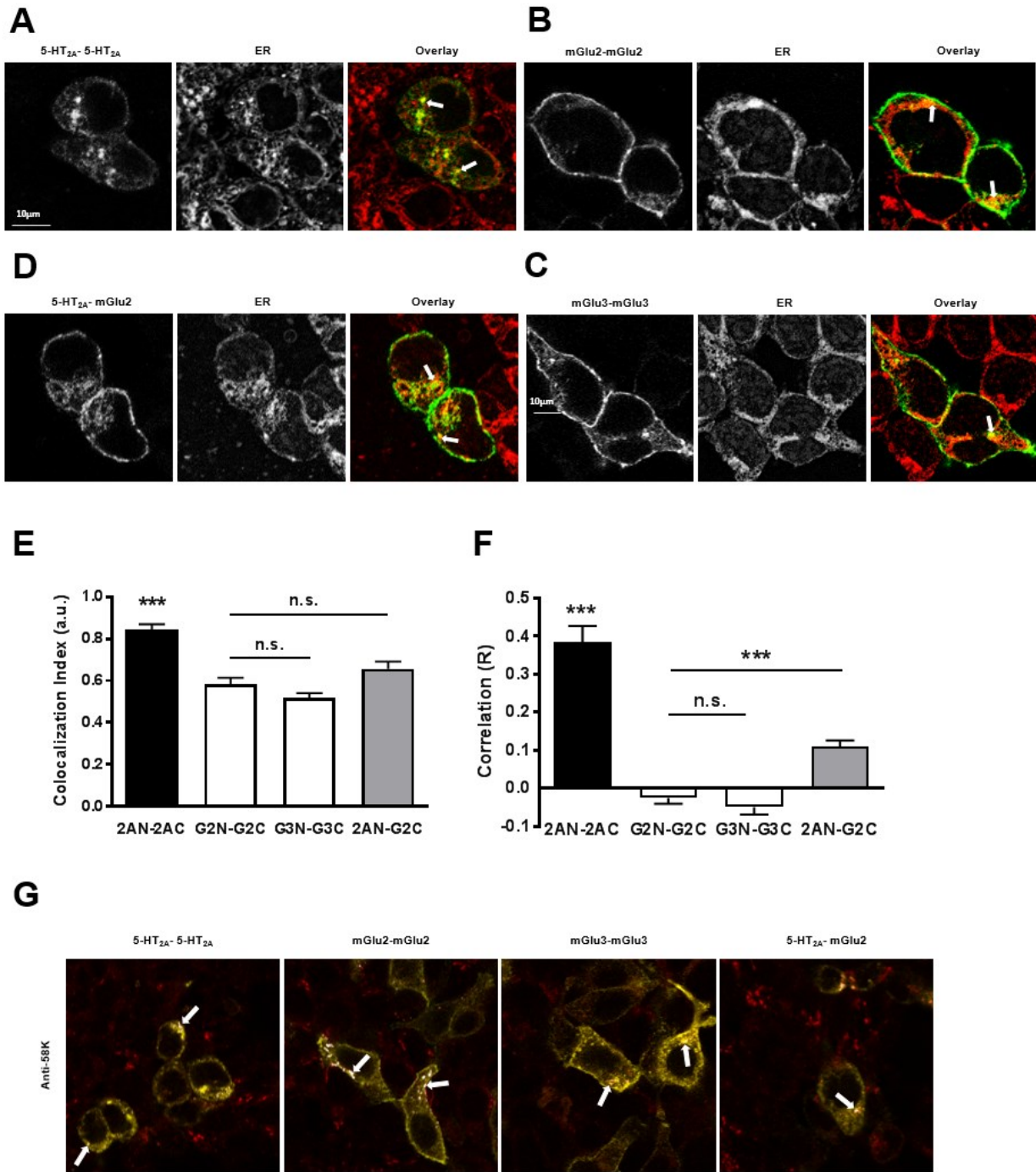
Fig. 10. Localization of mGluR2 is dysregulated in the frontal cortex of 5-HT_{2A}R^{-/-} mice. (A and B) Colocalization analysis of anti-mGluR2 and anti-Rab5 immunoreactivity in cortical primary cultures of 5-HT_{2A}R^{+/+} and 5-HT_{2A}R^{-/-} mice. Representative confocal micrographs (A). Manders' coefficient colocalization analysis (B) ($n = 30$ to 74 cell regions of interest in two independent experiments). Nuclei were stained in blue with Hoechst 33342. (C and D) Immunogold labeling for anti-mGluR2 immunoreactivity in the frontal cortex of 5-HT_{2A}R^{+/+} and 5-HT_{2A}R^{-/-} mice ($n = 4$ mice per genotype and 25 to 35 synapses per mouse). Representative photomicrographs showing excitatory synapses in mouse frontal cortex (C). Quantification of relative distribution of gold particles within postsynaptic spines (D). Data are means \pm SEM (B and D). Statistical analysis was performed using Student's t test (B) or two-way ANOVA with Bonferroni's post hoc test (D). * $P < 0.05$ and *** $P < 0.001$. Scale bars, $5 \mu\text{m}$ (A) and 100nm (C). n.s., not significant.

labeling. We focused our analysis solely on excitatory postsynaptic dendritic spines which in the frontal cortex region are the principal sites of glutamatergic input onto pyramidal neurons, and our previous data showed that 5-HT_{2A}R and mGluR2 crosstalk in this population of cortical cells. Within postsynaptic dendritic spines, particle clusters were observed at plasma membrane, inside the cytoplasm, or distributed on both plasmalemma and intracellularly (Fig. A3). For each synapse, distribution was scored based on these three categories. In neurons from 5-HT_{2A}R^{+/+} mice, the largest proportion of synapses showed both plasma membrane and intracellular labeling (Figs. 10, C and D). Additionally, there was a significantly greater proportion of gold particle clusters at the plasma membrane alone in neurons from 5-HT_{2A}R^{-/-} mice relative to 5-HT_{2A}R^{+/+} littermates (Figs. 10, C and D). Together, these findings indicate that subcellular localization of mGluR2 is affected by expression of 5-HT_{2A}R in mouse cortical pyramidal neurons.

XI. 5-HT_{2A}R-mGluR2 fluorescence complementation in the HEK293 maturation pathway

1. In the endoplasmic reticulum

To address whether homo or heterodimeric receptor's complexes can be matured in the ER, we assessed by confocal microscopy the colocalization and the codistribution of the BiFC signal with the ER dye ER-Tracker, using the same quantitative colocalization analyses. 5-HT_{2A}, mGlu2 and mGlu3 homodimers complementation signals overlapped with the red dye, suggesting post-translational assembling in this organelle (Fig.11A, B, C, E and F). More importantly, 5-HT_{2A}-mGlu2 BiFC signal colocalized with the reticulum (Fig. 11D). 5-HT_{2A}R homodimer exhibited a significantly higher index of colocalization index (Manders $p < 0.001$), accounting for $\pm 80\%$ of probes co-occurrence (Fig. 11E). No significant variation of mCitrine amount was measured (± 0.6) between the mGlu2 mGlu3 and 5-HT_{2A}-mGlu2 receptor formations (Fig. 11E). The 5-HT_{2A}R



(Fig 11)

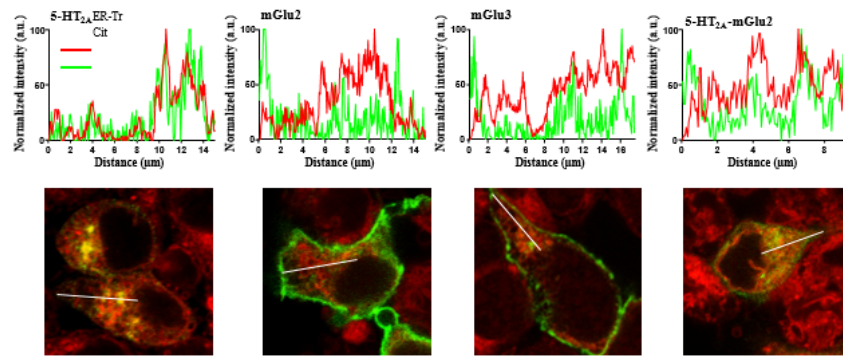
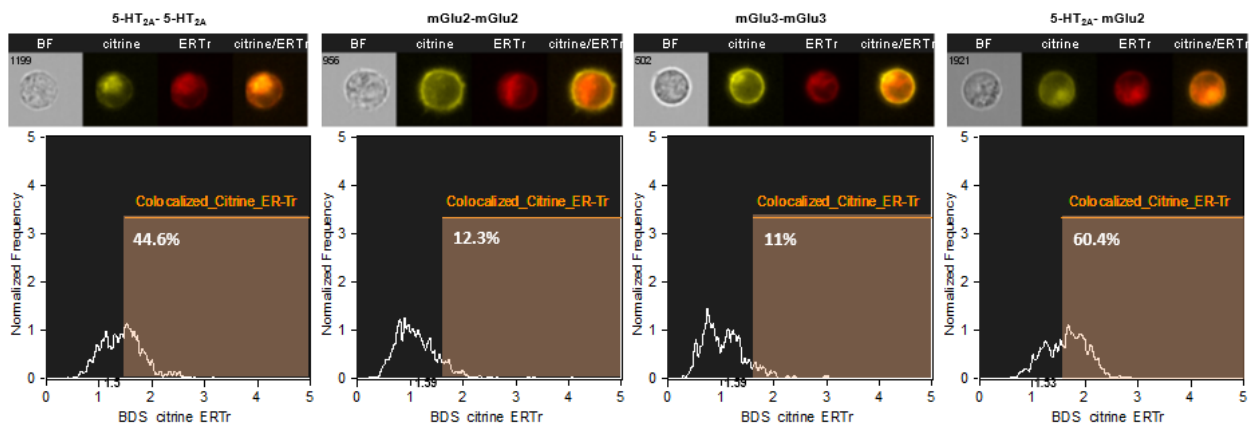
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Fig. 11. 5-HT_{2A}R-mGluR2 fluorescence complementation in the maturation pathway of HEK293. (A) Quantitative colocalization analysis of BiFC signals and the endoplasmic reticulum. (A to D) Single channel confocal micrographs of BiFC signal in HEK293 cells transfected to coexpress 5-HT_{2A}R-mCi-N172 and 5-HT_{2A}R-mCi-C67 (2AN-2AC), mGluR2-mCi-N172 and mGluR2-mCi-C67 (G2N-G2C), mGluR3-mCi-N172 and mGluR3-mCi-C67 (G3N-G3C), or 5-HT_{2A}R-mCi-N172 and mGluR2-mCi-C67 (2AN-G2C) and treated with the ER dye ER-Tracker. (E and F) Manders' and Pearson's coefficient colocalization analysis of BiFC mCitrine (yellow) and ER-Tracker (red) signal. (G) Colocalization between BiFC of 5-HT_{2A}R-mCi-N172 and 5-HT_{2A}R-mCi-C67, mGluR2-mCi-N172 and mGluR2-mCi-C67, mGluR3-mCi-N172 and mGluR3-mCi-C67, or 5-HT_{2A}R-mCi-N172 and mGluR2-mCi-C67 and the Golgi apparatus 58K. Immunofluorescence using a primary antibody against 58K protein and secondary AF568. Arrows point white pixels indicating positive signals for both probes. (H) Intensities distributions line scans from the membrane to the nucleus areas. Fluorescence intensity profiles illustrate the spatial codistribution of the two probes, BiFC and ER-Tracker. The relative similarity probes profiles in the case of 5-HT_{2A}R are consistent with relatively high and concentrated yellow pixels, as depicted by the image. mGluRs absence of paired profiles was reflected by absence of correlation. (I) ImageStream IFC-based BiFC of dual positive cell populations gated according to their Bright Details Similarity scores (BDS). Orange areas represent gated colocalized cell populations after indicated BDS calculation. Percentages of colocalized cells are shown. Images represent flowed cells and fluorescence profiles in each population acquired with a 60x objective. n=: 5-HT_{2A}: 579; mGlu2: 811; mGlu3: 281; 5-HT_{2A}-mGlu2: 974. Data are means ± SEM for 2 experiments (A to D, and I), one experiment (G). Statistical analysis was performed using one-way ANOVA with Bonferroni's *post hoc* test (E and F) and a Pearson logarithm transformation for the BDS score calculation (I). ***p<0.001, n.s. not significant. Scale bar is 10 μm.

homodimer elicited a high fractional overlap with the ER dye (Fig. 11E). Probes intensity codistribution analysis by Pearson correlation index (PCC) confirmed a relatively high index of codistribution between the signals, suggesting a linear correlation of their intensity in the organelle (Fig. 11F). This was supported by the intensity profiles plotted by line scans crossing the intracellular cell's area from the membrane to the nucleus (Fig. 11G). There was no such correlation in the case of the mGluRs (Fig. 11F). The PCC value of the 5-HT_{2A}-mGlu2 receptor heteromer with the ER dye was significantly higher than the mGluR homodimers but lower than the 5-HT_{2A}R homodimer, suggesting a partial overlap with the ER (Fig. 11F). Noticeably, the expression pattern of the heterocomplex was both plasma membranous and intracellular (Fig. 11D). Together, these colocalization analyses suggest that 5-HT_{2A} and mGluR2, come in close proximity within the ER.

2. In the Golgi apparatus

We used an immunofluorescence approach to detect the presence of the 5-HT_{2A}-mGlu2 receptor heteromer in the Golgi apparatus (GA) with an anti-58k antibodies targeting the resident protein 58K, conjugated to AlexaFluor568 secondary antibody. Due to the reduced area covered by the Golgi apparatus within cells compared to that of the ER, we qualitatively assessed the presence of the BiFC signal attesting the formation of the 5-HT_{2A}R-mGlu2 heterocomplex (Fig. 11H). Homomeric 5-HT_{2A} mGluR2 and mGlu3 receptors were found in small proportion in the Golgi apparatus, consistent with the upward flow of the protein biosynthesis (Fig. 11H). 5-HT_{2A}-mGlu2 heterocomplex colocalized with the GA. This suggests that the heterodimer may also be matured in the GA in relatively same proportion as the homomeric structures.

3. Image Flow Cytometry-based BiFC colocalization of 5-HT_{2A}-mGlu2 heterocomplex with the ER

In order to confirm previous findings in a larger cell populations, we combined IFC with BiFC in cells transfected with paired constructs. Double fluorescent populations were analyzed by the

Bright Details Similarity score (BDS). Negative gates were set for each complementation experiment by colocalizing each probe with the bright field. As previously described, a BDS score of >1.5 indicates highly colocalized cell populations²⁵⁴. All calculated control gates provided BDS scores ≥ 1.5 (Fig. 11G). This score delineate the gate over which cell population can be interpreted as highly colocalized. BDS score of 1.5 showed that 44,6% of 5-HT_{2A} BIFC-positive population were highly colocalizing with the ER. Scores of 1.593 and 1.594 gated mGluR2 and mGluR3 populations accounting for 12.3% and 11%, respectively. The heterodimer scored 1.532 with 60.4% of colocalized cells. Thus, this experiment distinguished consistent amount of highly colocalized cells populations and provided a similar trend between the BiFC constructs, although the analysis technique was different.

Discussion

It has long been known that coexpression of multiple 7TMRs by individual cells can affect processes related to agonist-promoted receptor signaling, endocytosis and down-regulation^{58,64,353-357}. The taste receptors provide a typical example. The finding that the class C heterodimer formed by T1R1 and T1R3 are sensors for the glutamate and aspartate amino-acid, commonly termed umami taste, while T1R2 and T1R3 associate to sense the sweet molecules, indicates that heteromerization is required for the perception of different taste stimuli. A change in the ligand binding domain (LBD) conformation relays an immediate restructuring of the dimeric interface, allowing to switch on downstream signals, which finally results in cell responses as diverse as taste perception can be³⁵⁸⁻³⁶⁰. Previous studies also suggested that for many documented 7TMRs heterocomplexes, such as δ -opioid- β_2 -adrenergic³⁶¹, α_{2A} adrenergic- β_1 -adrenergic⁵⁷, and adenosine A_{2A} -dopamine D_2 receptor³⁶², stimulation of only one of the components was sufficient to promote co-trafficking of the complex. Thus, the concept of 7TMRs co-trafficking in heterologous systems is not new. Pioneer observations suspected the co-internalization of two opioid receptors as a 7TMRs heterodimer, which were intensively studied for their pharmacology and their signaling as oligomers. The authors suggested that, etorphine treatment leads to internalization of δ -opioid receptor (δ OR) when expressed alone, but not when it is coexpressed with κ -opioid receptor (κ OR). However, they demonstrated that 30% of the surface fluorescence diminished indicating that δ OR homodimer can be internalized in cells expressing both δ OR and κ OR. It was then proposed that heterodimerization may participate in mGluR2 co-internalization⁶⁴.

In the present study, our data suggest that expression of 5-HT_{2A}R affects the subcellular distribution of mGluR2 through a molecular mechanism that involves a physical interaction of the two protomers. We further show that in the same cells, mGluR2 responds differently to processes related to agonist-induced trafficking through endosomal compartments when expressed with 5-

HT_{2A}R. In addition, in glutamatergic excitatory postsynaptic elements of *HTR2A*^{-/-} mouse frontal cortex, mGluR2 subcellular distribution is maintained close to the plasmalemma, similar to the prototypical localization observed in Flp-In T-REx stable HEK293 cells not induced to express 5-HT_{2A}R. Moreover, in cortical primary culture of 5-HT_{2A}R-deficient mice, the colocalization of mGluR2 and Rab5 is diminished, supporting the finding that 5-HT_{2A}R expression favors Rab5-mediated internalization mGluR2.

Under basal state experimental conditions (*i.e.*, the dialyzed FBS used in the culture media is free of the endogenous neurotransmitter 5-HT) our data validate the intriguing observation that 5-HT_{2A}R shows an elevated level of localization in intracellular vesicles, which could appear as an anomalous localization compared to most 7TMRs that are primarily addressed to the surface. This singular phenotype had previously been observed in several experimental systems, including HEK293 and CHO-K1 cells^{40,88,363}. By assessing, in intact COS-7 cells, the interaction between 5-HT_{2A}R and 5-HT_{2C}R's PSD-95/Disc-large/Zonula occludens-1 (PDZ) domains and co-transfected pre or postsynaptic PDZ resident proteins, it was reported a prominent intracellular presence of 5-HT_{2A}R when transfected alone, as a consequence of its interaction with specific sets of intracellular PDZ proteins³⁶⁴. Additionally, those protein sites together with PSD-95, being resident of the postsynaptic element, are in accordance with the main localization of 5-HT_{2A}R in this compartment (see below). Although not mutually exclusive, the physical interaction of 5-HT_{2A}R and PSD-95, besides providing structural localization, has also a functional role: PSD-95 enhances 5-HT_{2A}R-mediated activation of G α_q signaling as measured by IP accumulation in HEK 293 cells³⁶⁵. However, the direct interaction of 5-HT_{2A}R and PSD-95 accounting for this phenotype was interpreted in two ways: PSD-95 would anchor 5-HT_{2A}R in clusters to the cell surface, suggesting that its constitutive activity can be modulated by this postsynaptic proteins; and second, that this localization would favor interactions between 5-HT_{2A}R and other downstream effectors to elicit agonist-induced signaling yet restricting agonist-dependent internalization of 5-HT_{2A}R³⁶⁵; here,

PSD-95 inhibits agonist-induced internalization of 5-HT_{2A}R in order to maintain the receptor near cell surface pools. This process is also thought to participate, through the recognition by PSD-95 of specific PDZ motifs borne by 5-HT_{2A}R, in its proper targeting to dendrites of pyramidal neurons³⁶⁶. In support of such cellular organization, it was also proposed that, similar to the agonist-independent G protein activation of 5-HT_{2C}R, 5-HT_{2A}R may also have constitutive activity in heterologous systems, although to a much lesser extent than the former receptor, hence its intracellular localization³⁶⁷. Later, the constitutive activity of the 5-HT_{2A}R has been reevaluated: using a cell-based functional assay, a stronger turnover was measured, that could be modulated by mutating residues in the helices 6 and 3 (interaction model) of the WT receptor^{18,368}. Together, these reports in heterologous cellular systems addressed the nonstandard localization of 5-HT_{2A}R by providing functional and structural plausible explanations. Nonetheless, the 5-HT_{2A}R intracellular distribution is not restricted to such systems. Some of these regions contain neurons that express both 5-HT_{2A}R and mGluR2. While testing the subcellular distribution following the dendritic targeting mediated by the PDZ-binding domain of 5-HT_{2A}R, SFV-induced exogenous expression of 5-HT_{2A}R was found intracellularly, within dendrites of cortical primary neurons, in accordance with its endogenous distribution in culture³⁶⁶.

Immunocytochemistry studies on rat cerebellar cortex and olfactory bulb demonstrated both intracellular and membranous expression of the 5-HT_{2A}R protein using two different antibodies, targeting either the receptor N-terminus or the C-terminus (both tested and exhibiting an intracellular presence in form of puncta in S2 cells)³⁶⁹. In the rat spinal cord and ganglia, the immuno-targeted 5-HT_{2A}R was observed in small and large neurons and also interpreted as intracellular, *i.e.* in “dot-like” cytoplasmic distribution³⁷⁰. The receptor was also immuno-reactive in the somata and dendrites of Purkinje cells and in neural bodies of deep cellular nuclei, where again, we could observed dot-like cytosolic patterns¹³³.

In the rat VTA, another study showed the prominent cytoplasmic localization of 5-HT_{2A}R-like immunoreactivity colocalizing with the enzyme tyrosine hydroxylase, marker of dopaminergic neurons in this region, where 92% of the immunogold particles was detected. Neurons where TH was not detected also displayed this intracellular pattern, and was interpreted as membrane recycling of surface receptors, with all the caution attributed to immunogold assays¹²⁷. Given the highly probable constitutive activity of 5-HT_{2A}R (see above), it is possible to interpret this presence as agonist-independent activated receptor trafficking. Additionally, 5-HT_{2A}R immunoperoxidase stained other organelles such as the endoplasmic reticulum, Golgi apparatus, mitochondria and microtubules. This has also been observed in a study providing similar results in the rat cerebral cortex, using immunogold counterstaining and colocalization with the Microtubule Associated Protein 1A (MAP-1A)³⁷¹. The authors validated previous findings addressing the large intracellular presence of 5-HT_{2A}R in the rat CNS¹²³, and specified the possible link between the intracellular enrichment of 5-HT_{2A}R in small dendrites of pyramidal neurons and the microtubule network, as a likely interaction with MAP-1A. This interaction could potentially generate more interactions with intracellular signaling molecules, and could also suggest an intracellular signaling role or a dynamic association between surface and cytosolic 5-HT_{2A}R³⁷¹. A study focused on the caudate-putamen nucleus and the nucleus accumbens shell, attested that 5-HT_{2A}R and NMDAR colocalized in neuron's somata and dendrites. Interestingly, the intracellular presence of 5-HT_{2A}R was associated with cytoplasmic organelles rather than synaptic or extra-synaptic plasma membranes. Those neurons, assumed to be spiny striatal in the CPN and the NAc, were localized in dendrites and immuno-reacted with the endoplasmic reticulum and mitochondria. In the dorsolateral part of the CPN, labeling was more sharply localized in restricted areas between the plasma membrane and along mitochondria membranes, as well as in axon terminal endosomes, MVB, and small synaptic vesicles³⁷². In nociceptive neurons of rat lumbar dorsal root ganglia, 5-HT_{2A}R appeared in the cytoplasm, and at the junction of sensory and satellite cells, though also found at the plasma membrane of larger cell bodies³⁷³. While demonstrating the putative

interaction of 5-HT_{2C}R with 5-HT_{2A}R in the mPFC of rats, confocal images showed that in this critical region, 5-HT_{2A}R immunoreactivity was mostly intracellular³⁷⁴. In a study aiming to differentiate the anti-aversive action of serotonin inputs through activation of the 5-HT_{2A}R in the aversive system held within the midbrain periaqueductal grey matter, immunoreactivity was observed in both soma and dendrites of GABAergic interneurons³⁷⁵. The cytoplasmic localization of the 5-HT_{2A}R can also be found in unexpected cell types. Besides being observed in cell bodies and dendrites of pyramidal neurons in the cortex, the receptor was detected in Schwann cells using a newly developed monoclonal antibody targeting an epitope on the receptor N-terminal³⁷⁶. In rat hippocampus, 5-HT_{2A}R was detected in mossy fibers, indicating a presynaptic function of serotonin on glutamate axons. Additionally, the staining suggested that the receptor could be found on both excitatory and inhibitory neurons. Similar to the above findings, its intracellular presence was also recognized³⁷⁷. The approaches using immunoreactivity depends on the antibodies used, especially their reliability in terms of specificity³⁷⁸. It is possible, and often considered as a limitation, that several antibodies raised against different epitopes of the 5-HT_{2A}R receptor could lead to variable labelings of different cell types and subcellular localizations. However, the use of several antibodies at the same time followed by analysis of the matching profiles, allowed to validate and comfort the intracellular presence of the 5-HT_{2A}R³⁷⁹. The discrepancies could be attributed to the different conformations adopted by the receptor, *i.e.* different activation states that could reflect different levels of subcellular distribution: an antibody raised against the N-terminal end of the receptor would be likely inefficient to localized internalized vesicular receptors, because the N-terminal is in its lumen^{126,380}.

Conversely, the 5-HT_{2A}R was reported to be mostly located at the plasma membrane, in heterologous cell systems^{261,381}, or both intracellularly and at the cell surface¹³⁷. In mouse cortical neurons, both the dendritic plasma membrane and the axon cytoplasm were positive for the serotonin receptor²⁵². It is possible that the antibodies, used or the system itself, may contribute

to such variations in the distribution pattern of 5-HT_{2A}R. Another possibility is that, when the rat 5-HT_{2A}R is transfected in human cells, the trafficking proteins could not recognize its PDZ motif, which is different among species³⁸². *In vivo*, ultrastructural studies of the rat spinal cord demonstrated a major localization of the 5-HT_{2A}R at the plasma membrane in many subregions³⁸³. However, the same study found substantial staining in intracellular organelles, such as endosomes and MVB. Moreover, 5-HT_{2A}R may take distinct functions whether localize in the spinal cord, as opposed to the cerebral cortex or the midbrain. This could explain such differences in its expression. In a more recent review, it was proposed and admitted that the 5-HT_{2A}R can actually function and traffic differently, in pre or post synaptic terminals, as well as in different cell types³⁸⁴. The mixed plasma membrane/cytoplasm distribution pattern of 5-HT_{2A}R was also observed in the intestine³⁸⁵. In non-human primates, similar findings have been reported¹²⁴.

Together, this collection of studies indicates that the 5-HT_{2A}R may be found in multiple brain regions where it adopts mixed distribution patterns, and more importantly, corroborates the intracellular distribution of the receptor. In heterologous cell systems, either the plasma membranous or the cytoplasmic pattern can be due to the absence of specific trafficking and signaling interacting partners that are present endogenously *in vivo*³⁸⁶. The presence or absence of ligand could also cause discrepancies. Moreover, the specific localization of a brain region and/or or cell type, determines the receptor's function; this is another point that could explain its mixed distribution pattern. Lastly, the technique in use and how it is carried out could also affect the results. Several hypotheses have been proposed to explain such atypical behavior for a class A 7TMR, such as constitutive activity, intracellular signaling and as a sensor for serotonin concentration. In accordance with our findings herein, another possibility may be explored in future work. The basal activity of the 5-HT_{2A}R was substantial in the stable cell line used, and this seemed to relocalize mGluR2, in absence of agonist. While the activation of 5-HT_{2A}R implicated Rab5-mediated internalization of mGluR2, it is possible that the serotonin receptor plays a role in

the internalization of unbound interacting receptors and promote their agonist-independent internalization. This is consistent with the observation that in presence of mGluR2 agonist LY379268, its colocalization with Rab5 is reversed. This mechanism is mediated by its heteromerization with 5-HT_{2A}R. Another possibility is that the heteromeric formation would signal from intracellular compartments, offering a novel functionally never observed previously.

Most 7TMRs are trafficked through the endocytic pathway after internalization^{263,387}. We showed that, in the absence of 5-HT_{2A}R or mGluR2 agonists, the glutamate receptor colocalization indexes with endosome markers were higher when coexpressed with 5-HT_{2A}R, as compared to that obtained in HEK293 cells expressing mGluR2 alone. Our data also suggest that the presence of 5-HT_{2A}R in cells stably expressing mGluR2 increased mGluR2 colocalization with different markers of the endocytic pathway such as Rab5, Rab7, and the transferrin receptor. Considering that colocalization of mGluR2 and Rab5 was also reduced in *5-HT_{2A}R*^{-/-} mouse cortical primary cultures, these data provide evidence that 5-HT_{2A}R's constitutive activity affects the localization of mGluR2 within individual components of the endocytic pathway.

Although an important proportion of the 5-HT_{2A}R population is located intracellularly, we and others have validated the expected finding that addition of 5-HT_{2A}R agonists, including 5-HT or DOI, leads to activation of G α _{q/11} protein-dependent pathways, such as stimulation of phospholipase C (PLC) activity, which increases intracellular inositol triphosphate (IP3), diacylglycerol (DAG) and intracellular Ca²⁺ levels^{251,254,388}.

When HEK293 cells were transfected with both 5-HT_{2A}R-mCherry and mGluR2-mCitrine construct, we observed that mGluR2 is relocalized intracellularly like it is partially adopting the pattern of the serotonin receptor. However, this was not the case for all cells cotransfected with both constructs; while the 5-HT_{2A}R-mCherry was localized inside all the observed cells, mGluR2-eYFP was still maintained at the plasma membrane for some of them. It is likely that not all the cells express the same exact ratio of 5-HT_{2A}R/mGluR2 receptors. The relative stoichiometry, or

the amount of transfected DNA penetrating the cells in terms of copies, has been shown to affect the crosstalk between 5-HT_{2A}R and mGluR2, responsible for the mobilization of GIRK1/GIRK4 currents as a reporter of Gα_i and Gα_q activation. It has been already reported that in HEK 293 cells, the relative stoichiometry between the two cotransfected receptors is critical to elicit a robust crosstalk^{251,254}. Because the functional crosstalk has been validated by several functional assays (GIRK current and Ca²⁺ mobilization), in this study we selected clones that elicited a robust cross-signaling in addition to their intracellular colocalization. In transfected cells that express 5-HT_{2A}R-mCherry and mGluR2-eYFP, the exact number of inserted genes was not assessed, and it is possible that the relative stoichiometry between the two receptors was not conserved. Together, these observations provide an interesting conclusion, suggesting that, in cells, a characteristic stoichiometry is necessary to elicit both the functional crosstalk and the co-trafficking of 5-HT_{2A}R and mGluR2. Further work would be required to determine what is the exact relative stoichiometry in neurons co-expressing the two receptors and likely prone to cross-signal, and compare the findings to the number of exogenous DNA copies inserted into the cell genome of crosstalk positive clones.

These above considerations incline to speculate that a critical stoichiometry between 5-HT_{2A}R and mGluR2 can either be beneficial (*i.e.* physiologic) or detrimental (over/under-expressed) for the neuron and its neurotransmission, and that the intracellular localization of 5-HT_{2A}R is an important factor of regulation. This is relevant to schizophrenia. Following the simplest hypothesis that trafficking controls proteins' subcellular spatial and temporal distribution, it is then possible that a defect in this synchronicity may lead to unbalanced ratio of receptors, which, if maintained for a substantial amount of time and because of the entanglement of receptor trafficking and signaling, increase the likelihood to generate a global failure, in a form of a neuropsychiatric disorder.

The implication of subcellular trafficking in schizophrenia was the topic of several symposia where, for the first time, to our knowledge, links were made between gene association and molecular

mechanisms³⁸⁹. In particular, several themes were related to endosomal trafficking and clathrin-coated dependent endocytosis, as well as the *DTNBP1* gene³⁹⁰. In post mortem tissues extracted from subjects with psychotic disorders, altered levels of several proteins interacting with clathrin, AP-2 and dynamin-1, among others, have been found³⁹¹. These proteins regulate lysosome positioning and expression, endosomal trafficking and cell division (this review article lists several other proteins implicated in trafficking and detected in genetic or GWAS association studies). The *DTNBP1* gene is cited among several other primary candidates associated or altered in schizophrenia, such as *COMT*, *NRG1*, *RGS4*, *GRM3*, and *DISC1*^{143,392,393}.

Emerging hypotheses shedded light on a possible genetic dysregulation of the trafficking machinery and more specifically, the endosomal system, resulting in neurochemical and signaling abnormalities^{390,391}. It is supported by genome wide analyses that detected several genes implicated in schizophrenia related to the endosomal trafficking, and in particular to the potential defects of the Biogenesis of Lysosome-related Organelles Complex-1 (BLOC-1). The dystrobrevin-binding protein 1 (*DTNBP1*) gene that encodes the dysbindin-1 protein, together with *MUTED*, are essential components of the BLOC-1 protein complex, and has become a highly scrutinized gene for its implication in schizophrenia^{317,394}, as well as in cognition in healthy subjects³⁹⁵. Dysbindin is widely expressed in neurons, in both presynaptic terminal and postsynaptic densities throughout the brain^{395,396}. It is expressed in regions known to contain both 5-HT_{2A}R and mGluR2, such as the frontal and temporal cortexes, certain nuclei of the basal ganglia, the hippocampus, the thalamus, the amygdala, as well as the midbrain, which receives inputs from the claustrum that may control conditioning and sleep³⁹⁷, a region that also receives main serotonergic innervation from the DR³⁹⁸. Dysbindin mRNA is reduced in the dorsal prefrontal cortex and in the midbrain of patients with schizophrenia³⁹⁹. In the hippocampus of those patients, both mRNA and dysbindin protein are reduced. More importantly, this reduction in the contribution of dysbindin in the proper functioning of BLOC-1 has been shown to affect the surface

localization of D₂R, one of the main class A 7TMRs involved in both schizophrenia pathophysiology and its treatment. The dopamine hypothesis indeed postulates that, within the PFC, the D₂R-D₁R ratio is imbalanced in favor of D₂R, resulting in a reduction of the cortical excitability concomitant with a reduction of the inhibitory strength of GABA interneurons⁴⁰⁰. In human SH-SY5Y neuroblastoma cells and in cultured cortical neurons, the reduction in dysbindin expression has been shown to augment the cell surface presence of D₂R, but not D₁R⁴⁰¹. siRNA-induced downregulation of dysbindin decreased levels of SNAP25 and synapsin I, together with a reduction of glutamate release, suggesting that dysbindin plays a role in exocytosis of glutamate-containing synaptic vesicles⁴⁰². Similar findings have also been reported in the *sandy* mouse (a natural occurring null mutation of *DTNBP1*)⁴⁰³. Other components of the BLOC-1, such as snapin and pallidin, can respectively interact with SNAP25 and syntaxin 13⁴⁰¹, two members of the SNARE family, master regulators of membrane fusion^{404,405}. It has also been proposed that, under elevated synaptic activity, pallidin interacts with Rab5 to recycle synaptic vesicles more efficiently in pools within early endosomes. Interestingly, in yeast, BLOC-1 may also regulate and participate to the early endosome maturation by interacting with Rab5 homolog⁴⁰⁶. These interactions, if altered, can also affect the dynamic and the spatial distribution of endosomes by disturbing the actin/microtubule cytoskeleton structure⁴⁰⁷. In the case of dysbindin, it has also been shown that the protein does not primarily regulate the endocytic trafficking, but rather influences later steps that lead to degradation. The overcharge of D₂R at the cell surface is a consequence of an inefficient degradation capacity. Additionally, the authors also suggest that this role of dysbindin is perhaps not strictly specific to D₂R but also to other 7TMRs, in various cell types⁴⁰⁸. The presynaptic reduction of dysbindin was also correlated with an augmentation of VGLUT-1 in the hippocampus of more than 75% of the studied schizophrenia population. It has thus been suspected that, because altered DTNBP1 in presynaptic glutamatergic terminals can cause BLOC-1 to dysfunction, the augmentation of VGLUT-1 can result from a lesser degradative

function of BLOC-1, thus providing a plausible cause of glutamate dysfunction in schizophrenia^{399,409,410}.

It is then tempting to speculate that a defect in receptor stoichiometry, for instance 5-HT_{2A}R and mGluR2, due to a defect in protein trafficking/signaling, and/or vice-versa, is a plausible explanation for the dysregulation observed in patients with schizophrenia syndrome¹⁸³. What could be at the origin of such a breakdown of the trafficking system is beyond the scope of this study; it is possible that persistent epigenetic alterations can cause such deleterious effects in the core machinery of protein synthesis and recycling^{411,412}.

Many neuropsychiatric disorders, including schizophrenia, could be caused by alterations of the dopaminergic neurotransmission and related subcellular signaling⁴¹³. Hyperdopaminergic activation in the prefrontal cortex (*i.e.*, increased D₂/D₁ ratio) associated with a lack of dopamine tone in the striatum, may account for both positive and negative symptoms of the schizophrenia syndrome^{414–416}. The actual mechanism is complex but one model described that in dysbindin-deficient primary cortical neurons of *sandy* mice, BLOC-1-mediated trafficking of D₂R toward its degradation following ligand binding, corresponded to a reduction of dopamine-induced subcellular internalization of D₂R. As a result, abundant D₂R receptors at the cell surface seemed to traffic much faster than D₁R⁴⁰⁰. This excess of signaling has deleterious effects on the GABAergic neurons by reducing their excitability in the PFC and in the striatum, and more precisely, in the layer V of the PFC pyramidal neurons⁴⁰⁰. These defects could be the results of the dopamine neurotransmission imbalance observed in schizophrenia⁴⁰⁰. First generation antipsychotics have high affinity for the D₂R, although atypical antipsychotics target the receptor as well^{309,314,415,417,418}.

In the case of a defect in the 5-HT_{2A}R trafficking in the PFC, similar to the effects described above on D₂R, it is possible that a disruption of the constitutive activity of 5-HT_{2A}R, either by a defect of the endocytosis or the degradation processes, could lead to receptor overexpression and because

5-HT_{2A}R seemed to co-internalize mGluR2, the latter would also be downregulated, resulting in the loss of inhibition of glutamatergic transmission by the PFC neurons. This is consistent with the fact that agonists of mGluR2 have a potential antipsychotic activity. Thus, it would be interesting to search into the relationship between 5-HT_{2A}R mGluR2 and DTNBP1, although a study has implicated 5-HT_{2A}R and dysbindin in humans⁴¹⁹. This is supported by the fact that the involvement of the *HTR2A* gene in the physiopathology of schizophrenia is not clear. Since neither apparent gene mutations, nor alteration of the receptor binding properties have been clearly demonstrated, it is possible to speculate that the regulation of the receptor is involved, rather than structural impairments of the peptide⁴²⁰.

The potential role of heteromerization as the mechanism underlying the effect of 5-HT_{2A}R on subcellular localization of mGluR2 is supported by our findings demonstrating intracellular localization of the heterocomplex by BiFC, PLA and FRET. Surprisingly, we could not detect any FRET signal nor PLA association near the plasma membrane. As above-mentioned, most of the 5-HT_{2A}R seems located intracellularly, and despite being able to elicit a fast calcium mobilization, validating the fact that 5-HT_{2A}R can signal from the plasma membrane (as it is difficult to imagine that a monoamine like 5-HT crosses the plasma membrane and binds intracellular receptors), there was low to no interactions in this area. There was though a very faint eCFP fluorescence at the plasma membrane. This is puzzling and somewhat not consistent with the standard localization of most 7TMRs. If these findings are consistent, it will be necessary to further examine the role of receptor heteromers in the intracellular trafficking and signaling of 7TMRs.

Recent findings point towards instability of a family A 7TMR oligomeric interface^{47,48,81}. Our current data demonstrating that the assembly of 5-HT_{2A}R and mGluR2 is able to modulate and affect dramatically the distribution of one of them can hardly be explained by a transient interaction lasting fractions of seconds as it has been demonstrated for other class A 7TMRs (kiss-and-fly model)^{31,421-423}. Here, the interclass 7TMR heteromeric assembly supports the concept that 5-

HT_{2A}R and mGluR2 are part of a stable receptor complex structure that is co-trafficked and can cross-signal consistently along different experiments^{251,253,254,424}. Our findings in the maturation pathway, suggesting that 5-HT_{2A}R and mGluR2 can come together in close enough proximity to be detected by BiFC confocal microscopy support that, at least in these compartments, the existence of a stable heteromer is conceivable. Further work, however, will be necessary to fully characterize the dynamics of this structural assembly. Given the active and structurally preferential TM domain involved in the homodimerization of mGluR2/3 and 5-HT_{2A}R, a recent photocrosslinking study addressed with high accuracy the potential TM domain at the interface of the heterocomplex formed by 5-HT_{2A}R and mGluR2⁸⁹. This indicates a potential site of stabilization that would explain herein results especially in the surprising case of DOI activating 5-HT_{2A}R-dependent trafficking of mGluR2 in Rab5 endosomes. Although previously thought as stable, the μ -opioid receptor dimer has recently been observed as a minor and negligible entity⁵¹. Whether 5-HT_{2A}R association with mGluR2 it is stable or not, and whether this is a limitation, has to be addressed by additional experiments, such as single-molecule pull-down assay (SiMPull) assay^{425–427}.

An interesting observation was that whereas DOX-induced expression of 5-HT_{2A}R augments the density of mGluR2, but not that of mGluR3 or mGluR2 Δ TM4, G $\alpha_{i/o}$ protein coupling induced upon activation of mGluR2 was substantially reduced in DOX(+) cells as compared to DOX(-) cells. Although this finding further supports specificity of the cross-talk between 5-HT_{2A}R and mGluR2, the exact mechanism remains to be investigated. Nevertheless, a potential explanation to reconcile these results is the existence of a compensatory pathway to rescue mGluR2-dependent function due to the higher degree of mGluR2 intracellular localization upon DOX-induced 5-HT_{2A}R expression.

Acting through 5-HT_{2A}R, LY379268 and DOI differently affected the distribution of mGluR2 within Rab5-positive endocytic vesicles, as well as the physical interaction between 5-HT_{2A}R and

mGluR2. Thus, we found that whereas coexpression of 5-HT_{2A}R prevented the effect of mGluR2 agonists on both mGluR2 endocytosis and down-regulation, agonist-induced activation of mGluR2 decreased its physical proximity with 5-HT_{2A}R. Our data also support the conclusion that agonist activation of 5-HT_{2A}R increased the colocalization between mGluR2 and Rab5. This effect required 5-HT_{2A}R–mGluR2 heteromeric assembly because it was reduced by the interfering peptide TAT-TM4. However, under the same experimental conditions, and opposite to the effects of LY379268, both mGluR2 density and 5-HT_{2A}R–mGluR2 heteromerization remained unchanged. These data suggest that whereas DOI augments intracellular distribution of mGluR2 within Rab5-positive and clathrin-dependent endosomes coexpressing 5-HT_{2A}R and mGluR2, internalized mGluR2 is not degraded after entering the late endosomal/lysosomal pathway. Sorting of mGluR2 into the recycling pathway could provide a potential explanation. Additional work will be necessary to determine the population of intracellular vesicles downstream of Rab5-positive endosomes responsible for increased endocytic uptake of mGluR2 without receptor down-regulation.

In the absence of DOI, colocalization of mGluR2 and Rab5 was comparable between cells exposed to either TAT-TM4 or the TAT-tagged scrambled peptide. This absence of effect of TAT-TM4 on colocalization of mGluR2 and Rab5 at steady-state conditions does not call into question the capability of the TAT-tagged peptide to reduce 5-HT_{2A}R–mGluR2 complex assembly. Instead, together with the effect of TAT-TM4 preventing DOI-induced augmentation of mGluR2 localization within Rab5-positive endosomes, these findings suggest that, in the absence of exogenous agonists such as DOI, disruption of 5-HT_{2A}R–mGluR2 heteromerization does not imply rapid mGluR2 trafficking from Rab5-positive endosomes to alternative endocytic compartments.

Although discovered in the late 1950's⁴²⁸, clozapine still remains the gold standard for schizophrenia treatment⁴²⁹. It binds with relatively low affinity to dopamine D₂R and other monoaminergic neurotransmitter receptors, but similarly to other atypical antipsychotics, clozapine

shows a particularly high affinity for 5-HT_{2A}R^{430,431}. Activation of mGluR2 by mGluR2/3 agonists, including pomaglmetad or LY2140023 (an oral prodrug of LY404039), has been proposed to treat tripartite symptoms in patients with schizophrenia²²⁶, but pomaglmetad did not show enough clinical utility⁴³². We previously proposed that chronic treatment with clozapine and other atypical antipsychotics induces a selective down-regulation of *mGluR2* expression through an epigenetic mechanism involving repressive histone modifications at the promoter region of the *mGluR2* (*GRM2*) gene in mouse and human frontal cortex^{433,434}. These preclinical findings were validated by clinical work showing the absence of therapeutic effects of pomaglmetad in patients with schizophrenia previously treated with atypical antipsychotic medications⁴³⁵. Our current findings here propose an alternative, although not mutually exclusive, explanation by which long-lasting exposure to clozapine down-regulates mGluR2 density through a mechanism that requires its heteromerization with the 5-HT_{2A}R. We observed that subcellular colocalization between 5-HT_{2A}R and mGluR2 was reduced upon clozapine exposure. This effect was not induced by overnight exposure to M100907, was not observed in cells stably expressing mGluR2ΔTM4 and induced to express 5-HT_{2A}R, and was prevented by a TAT-tagged peptide that disrupts 5-HT_{2A}R–mGluR2 heteromerization. Similarly, clozapine, but not M100907, reduced total mGluR2 density and mGluR2 localization at both cell surface and in intracellular compartments. Thus, continued exposure to clozapine, but not M100907, affects mGluR2's subcellular distribution and density through a mechanism that requires 5-HT_{2A}R–mGluR2 complex formation. Further work will be necessary to better understand the trafficking pathway responsible for alterations in mGluR2 cellular distribution among 5-HT_{2A}R–positive intracellular vesicles and plasma membrane.

In the perspective of studying repressive epigenetic mechanisms at the *mGlu2* promoter, our lab showed that in mouse frontal cortex lacking the *HTR2A* gene, the mRNA level of *mGlu2* are also decreased, whereas 5-HT_{2C} and *mGlu2* levels are not affected. Although these findings demonstrate in animal model an effect of 5-HT_{2A}R affecting mGlu2 at the transcription level, our

results in a cellular system showed that expression of 5-HT_{2A}R have a tendency to upregulate mGluR2 at the protein level. Although these two observations are distant to the mechanism of action, both seem to be consistent, although there is no evidence yet to prove it.

Exposure of GF-62 cells to 5-HT_{2A}R antagonists, including clozapine, has been shown to internalize the receptor which decreased its cell surface density. Similarly, an increase in the intracellular concentration of 5-HT_{2A}R in apical dendrites of rat pyramidal cells has been reported, following exposure to such drugs. An alternative mechanism to ligand-induced dynamin dependent endocytosis has been proposed, involving caveolae⁴³⁶. In the present study, we observed that in HEK293 cells induced to express 5-HT_{2A}R, several inverse agonists/antagonists relocated the receptor intracellular distribution to the plasma membrane area. Although it is not clear whether the receptor is actually spanning the membrane or stationing underneath in endosomes, only clozapine treatments seemingly failed to relocate the receptor. Even more surprising was that olanzapine, a clozapine analog, did affect 5-HT_{2A}R localization. Such puzzling finding would require fine investigations on how antagonists or inverse agonists affect the constitutive activity of 5-HT_{2A}R. The constitutive activity of 5-HT_{2A}R can affect ritanserin potency¹⁸. It is also a property of inverse agonists to affect the basal activity of 5-HT_{2A}R, while antagonist compounds should remain “inactive”²². A study *in vivo* provided more insights into this particular mode of action typical to 5-HT_{2A}R⁴³⁷.

We were unable to characterize the function of our mGluR3 clones with the GTPγS assay. This is the reason why HEK293 were transfected with the chimeric G protein qi9, in order to test intracellular calcium release. The positive signal elicited by mGlu3 clones suggested that the cell line held a functional receptor. To note, we observed a large presence of intracellular eYFP-positive vesicles, attesting of a constitutive activity of the mGlu3 receptor. In the second step, we recently addressed the reason why mGluR3 was not able to efficiently mobilize Gα_i protein signaling. In several brain tissues, GTPγS assays were performed in presence of the NAM

mGluR3. Robust signal was elicited accounting for the presence of mGluR2 and mGluR3 composite signals. Application of NAM reduced the signal elicited by LY379268 binding in the GTPγS readout (data not shown, in progress). Together, this indicates that mGluR3 can efficiently couple to G proteins, but for an unknown reason, this was not the case in our cell line. A consistent analogy is provided by a study that was able to distinguish mGluR2 signaling from mGluR3²³⁰.

Considering our findings in this heterologous cellular system, the fact that over expression or overactivation of the 5-HT_{2A}R may as well induce over internalization of mGluR2 into more internalized compartment is consistent with a defect of activity. It would reduce the surface availability and the overall density of mGluR2, and likely damped the effect of antipsychotic glutamate agonists such as pomaglumetad. Our findings showed that coexpression of 5-HT_{2A}R and mGluR2 elicited some populations of more internal glutamate receptors, whereas as in average, unlike WT, 5-HT_{2A}R^{-/-} mice postsynaptic area, mGluR2 tends to be nearby the plasmalemma.

Concluding remarks

Although some reported examples may reflect data overinterpretation or ambiguity due to the system in use, the concept that class A 7TMRs form receptor complexes is supported by numerous experimental approaches. As of yet, the functional importance of 7TMRs oligomerization remains controversial. Our data obtained with stable expression of mGluR2 or mGluR2/mGluR3 chimeric constructs and TAT-tagged peptides to disrupt 5-HT_{2A}R–mGluR2 complex formation support the conclusion that 5-HT_{2A}R affects the subcellular localization of mGluR2 through 7TMR heteromerization. The validity of this concept is further advanced by our findings showing alterations in mGluR2 cellular distribution in frontal cortex pyramidal neurons of 5-HT_{2A}R^{-/-} mice as compared to control littermates. In short, these findings illuminate the importance of interclass heteromerization on 7TMRs localization and trafficking.

Limitations

The relatively low percentage of FRET efficiency is also consistent with the relative proportion but also the orientation of the fluorescent proteins. As we cannot establish a positive control to detect what is the maximum efficiency of this system, we could not compare our signal to a standard maximum FRET_e. However, we showed that the mGluR2 Δ TM4-eYFP chimera FRET_e was reduced by 2 folds. This indicates that the interaction is less stable with the mutant or alternatively, we cannot rule out that the mutant's fluorescent protein is orientated differently. Orientation of the fluorescent protein is critical for the FRET to occur and its efficiency. Another possibility is that, as the KD of the mutant is higher than the WT (Table 1), the function of this receptor is affected and could not change its conformation as the WT does.

Another important question was the origin of mGluR2 trafficked by 5-HT_{2A}R. We showed that the application of the dynamin inhibitor dynasore prevented endocytosis of mGluR2 when 5-HT_{2A}R was coexpressed. Additionally, BiFC and FACS experiments suggested that the 5-HT_{2A}-mGlu2 receptor heteromer could be synthesized in the ER, and also detected in the Golgi apparatus. Several hypotheses can be provided: the heterocomplex is stable from its biosynthesis in the ER to the plasma membrane and it is trafficked as a stable entity; partially synthesized in the ER and partially assembled later at the cell surface or in endosomes; or mGlu2 provides an ER exit routes for 5-HT_{2A}R, similar to the GABA_BR or β_2 AR, and the association is dynamic during the trafficking or upon ligand-induced G protein activation. An known limitation to those findings using the BiFC assay, is that complements of the fluorescent protein can be covalently fused and therefore, the heteromeric association is irreversible^{438,439}. This has limited our studies to the maturation pathway, which suggested, even if the association is irreversible, that a close contact occurred. Additional work would be necessary to delineate and ascertain the directionality and the timeline of this association, for example by tracking 5-HT_{2A}R biosynthesis and trafficking in mGluR2^{-/-} mice.

Although the HEK293 cells have been used extensively and reliably over the past decades as a neuronal model substitute, they are not neurons. If they do not express mGluR2 and 5-HT_{2A}R, they still translate 7TMR interacting proteins, scaffolds and chaperones necessary for endogenous cellular operations and contain the basic machinery to robustly allow and maintain the structure and the function of overexpressed 7TMRs, or other transfected or stable receptors and transporters. It is interesting to have in mind the native receptor expressed by this cell line and control the expression of close and studied receptors to avoid any interference⁴⁴⁰. There is no serotonin in our DMEM, although serotonin is an important neurotransmitter and a hormone for the kidney function. *GRM2* and *GRM3* are also absent though it seems that *GRM2* has traces as assessed by a microarray study⁴⁴⁰.

The question about the affinity of the mGlu2 chimera compared to the WT mGluR2 has not been addressed in this study. Whether the difference in affinity comes from the impaired association with 5-HT_{2A}R or the mutant by itself, constitutes a point to address.

Colocalization analyses sometime yielded high variances among and within experiments. This is perhaps due to the fact that not all cells elicit the same amount of 5-HT_{2A}R-eCFP after being induced, and because stoichiometry seems important, this can lead to a differential internalization of mGluR2 consistent with the level of 5-HT_{2A}R in Rab5 or Rab7.

The primary cortical cultured neurons were extracted from embryonic mice. Because we used the total cortical area, the exact nature of the neurons analyzed by immunofluorescence is not known, although we favored pyramidal shaped neurons in our collection.

The Pearson correlation coefficient is sensitive to the fluorescence intensity because the correlation is maximal when intensities are linearly associated. The use of Manders coefficient was more appropriate in those cases, and explains why the tandem was not systematically used in this study.

Table 1**[³H]LY341495 binding saturation curves in Flp-In T-REx HEK293 cells**

	DOX(-)		DOX(+)	
	K _D	B _{max} (pmol/mg prot)	K _D	B _{max} (pmol/mg prot)
mGluR2-eYFP	1.59 ± 0.21	36.94 ± 1.43	2.27 ± 0.15	51.81 ± 1.11
mGluR3-eYFP	1.16 ± 0.24	5.47 ± 0.30	0.68 ± 0.08	5.56 ± 0.16
mGluR2ΔTM4-eYFP	89.4 ± 4.84	2.33 ± 0.38	105.4 ± 4.03	3.77 ± 0.38

[³H]LY341495 binding saturation curves in Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP, mGluR3-eYFP or mGluR2ΔTM40-eYFP and harboring 5-HT_{2A}R-eCFP at the inducible locus. Cells were untreated [DOX(-)] or treated with doxycycline [DOX(+)] (n = 2). The data are presented as the mean ± s.e.

[³H]ketanserin binding saturation curves in Flp-In T-REx HEK293 cells

	DOX(-)		DOX(+)	
	K _D	B _{max} (fmol/mg prot)	K _D	B _{max} (fmol/mg prot)
mGluR2-eYFP	n/a	n/a	0.65 ± 0.07	3211 ± 115.9
mGluR3-eYFP	n/a	n/a	0.64 ± 0.09	3343 ± 153.7
mGluR2ΔTM4-eYFP	n/a	n/a	0.74 ± 0.12	7923 ± 416.0

[³H]Ketanserin binding saturation curves in Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP, mGluR3-eYFP or mGluR2ΔTM40-eYFP and harboring 5-HT_{2A}R-eCFP at the inducible locus. Cells were untreated [DOX(-)] or treated with doxycycline [DOX(+)] (n = 2). The data are presented as the mean ± s.e. n/a, not applicable.

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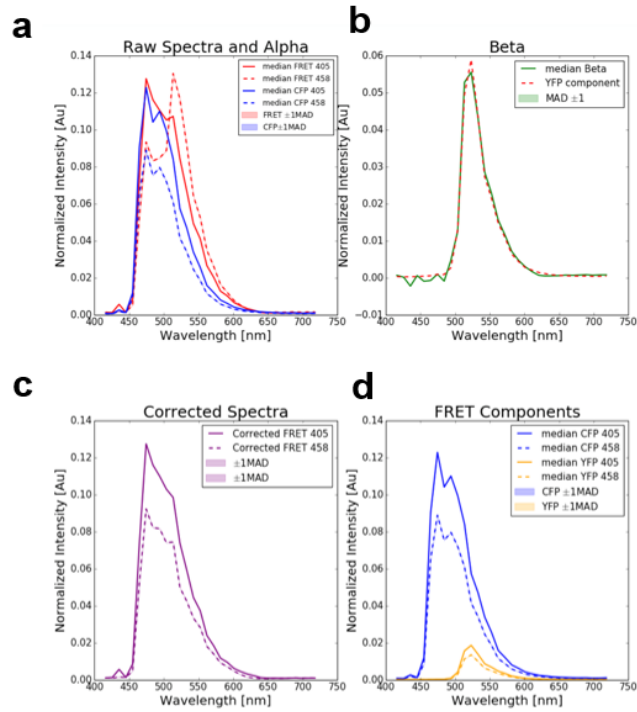
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VITA

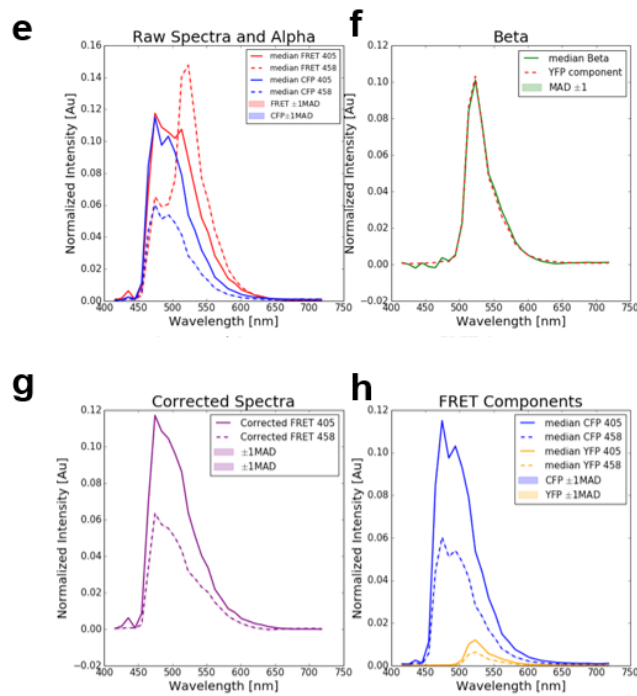
Rudy Toneatti was born on June 10th, 1977 in Paris, France. He is a French citizen and spent about four years working and studying at the VCU department of Physiology and Biophysics. He received a Bachelor of Art in Philosophy at the Nice University after being graduated with a *Baccalauréat* in Math, Physics and Biology. He also obtained a Master in Social and Political Sciences at the Sorbonne University in Paris. He did a Master in Integrative Biology and completed a first internship in Paris at the *Centre du Fer à Moulin* under the supervision of Dr Marta Garcia on tracking mitochondria trafficking along genetically modified mice piriform cortex neurons. Then travelled to Richmond for completion of the second graduating internship. After a year as a research assistant in the González-Maeso's Lab, he entered the Graduate School at the VCU School of Medicine where he studied for the obtainment of a PhD in Neuroscience.

APPENDIX

mGluR2-eYFP

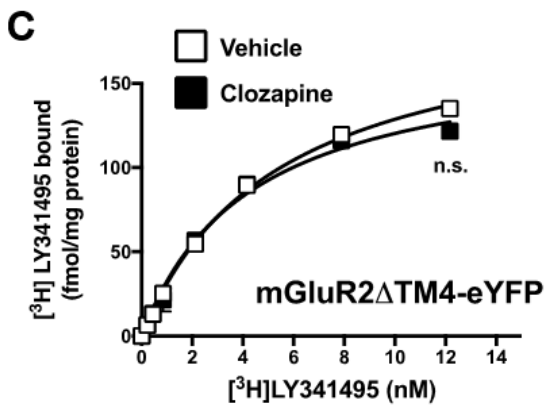
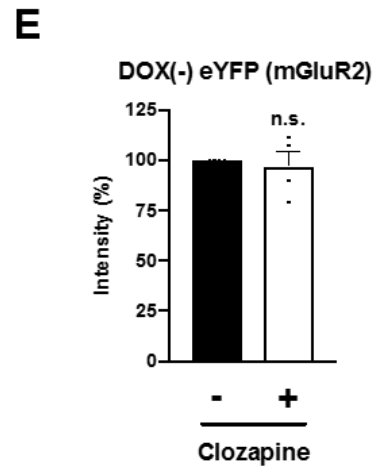
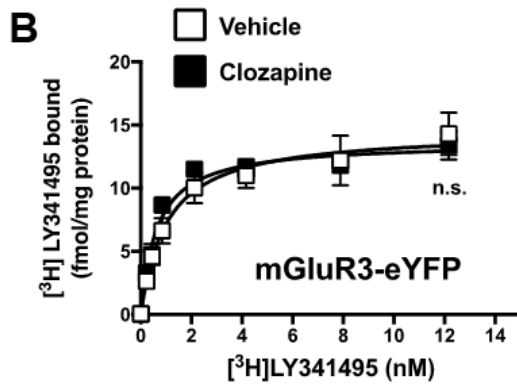
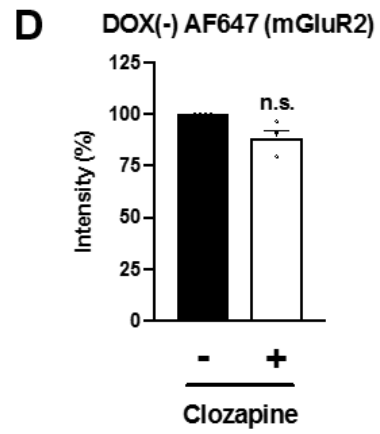
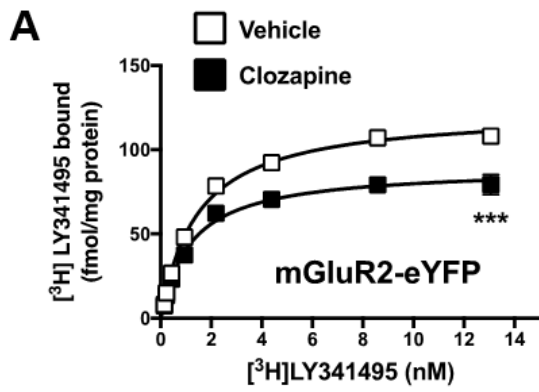


mGluR2 Δ TM4-eYFP



(Fig. Appendix .1)

Fig. A1. Average spectra and sensorFRET analysis for Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP or mGluR2 Δ TM4-eYFP, and harboring 5-HT_{2A}R-eCFP at the inducible locus. (**A** and **E**) Linear unmixing of the eCFP (donor) component from the raw spectra, the ratio of which provides the alpha parameter used in the sensorFRET analysis. (**B** and **F**) Fitting of the β parameter, which is the difference between the 405 and 458 nm spectra after scaling 405 by α . (**C** and **G**) Raw spectra at both frequencies after the acceptor direct excitation component is removed using β^{339} . (**D** and **H**) Linear unmixing of the donor and acceptor from the corrected spectra, where the FRET efficiency is proportional to the ratio of acceptor magnitude to donor magnitude. It can be seen that the ratio of eYFP magnitude to eCFP magnitude is larger in the mGluR2-eYFP group (indicating a higher FRET efficiency) compared to the mGluR2 Δ TM4-eYFP group.



(Fig. Appendix .2)

Fig. A2. Effect of clozapine on mGluRs density. **(A to C)** Flp-In T-REx HEK293 cells stably expressing mGluR2-eYFP **(A)**, mGluR3-eYFP **(B)** or mGluR2 Δ TM4-eYFP **(C)** and harboring 5-HT_{2A}R-eCFP at the inducible locus were treated with doxycycline, exposed overnight to clozapine (10 μ M), or vehicle, and harvested to perform radioligand binding assays with the mGluR2/3 antagonist [³H]LY341495 (n = 4 independent groups of membrane preparations). Cell surface localization of mGluR2-eYFP with an Alexa Fluor 647 (AF647)-tagged antibody **(D)** and total mGluR2-eYFP signal **(E)** density were assessed by flow cytometry assays (n = 3 – 5 independent experiments). Data are mean \pm SEM. Statistical analysis was performed using the *F* test. ****P* < 0.001, n.s., not significant.

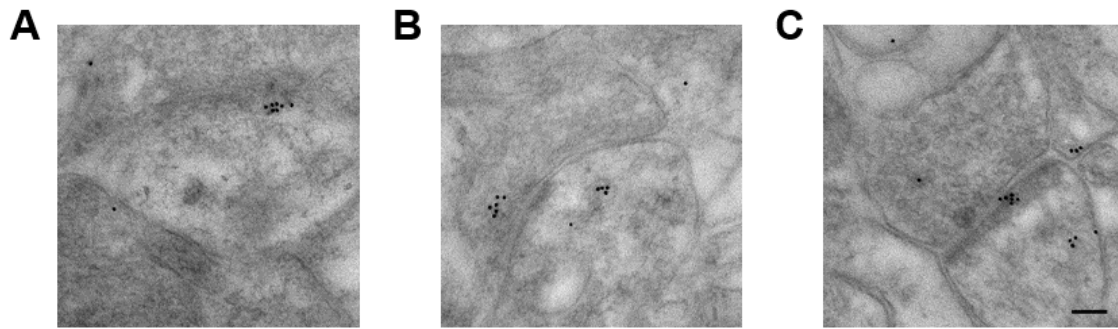


Fig. A3. Representative photomicrographs showing excitatory synapses in mouse frontal cortex scored as membrane only (**A**), intracellular only (**B**), or both (**C**) based on postsynaptic distribution. Scale bars, 100 nm.

(Fig. Appendix .3)

