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Exploring the Heteromeric Interface of the 5-HT_{2A}-mGlu2 Receptor Complex

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science in Physiology and Biophysics at Virginia Commonwealth University

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List of Abbreviations

5-HT	Serotonin Receptor
5-HT _{2A}	Serotonin Receptor 2A Subtype
5-HT _{2C}	Serotonin Receptor 2C Subtype
APD	Antipsychotic Drug
BSA	Bovine Serum Albumin
Co-IP	Coimmunoprecipitation
D1/D2	Dopamine D1/ D2 receptor
dFBS	Dialyzed Fetal Bovine Serum
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
EPS	Extrapyramidal Side Effect
GPCR	G protein Coupled Receptor
LSD	Lysergic Acid Diethylamide
mGlu2	Metabotropic Glutamate Receptor
PBST	Phosphate-Buffered Saline + 0.1% Tween 20
SDS-PAGE	Sodium dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TBST	TRIS-Buffered Saline + 0.1% Tween 20
TRS	Treatment Resistant Schizophrenia
UAA	Unnatural Amino Acid
WT	Wild-Type (Control)

Abstract

Schizophrenia is a serious mental disorder characteristic of several neurotransmitters including dopamine, serotonin, and glutamate being in imbalance. Early therapies focused solely on dopamine antagonism and second-generation antipsychotics focused on the dopamine and serotonin systems and their respective G protein coupled receptor (GPCR) proteins. Although debate for dimerization of certain classes of GPCR exist, the establishment of an mGlu2-5-HT_{2A} heterocomplex, which is implicated in schizophrenia is of interest. Previous studies have used a mutation-based approach to identify transmembrane domain 4 (TM4) as the domain responsible in mGlu2 for mediating heteromerization before narrowing down the individual amino acids responsible for the interface. A similar approach to consider which portion of 5-HT_{2A} is responsible for the heterodimeric interface was used in this study. We confirm that wild type 5-HT_{2A} and mGlu2 coimmunoprecipitate. Two 5-HT_{2A} chimeric constructs involving full and partial N terminal half TM4 mutations were created using aligned residues of 5-HT_{2C} (which is unable to dimerize with mGlu2). In addition to providing valuable insight into the structural arrangement of GPCR heteromers involving different families, these findings offer future direction for photo-crosslinking experiments aiming to identify individual amino acids within the 5-HT_{2A} responsible for mediating the heteromeric interface of the mGlu2-5-HT_{2A} complex.

Introduction

Schizophrenia

Schizophrenia is a debilitating and deteriorating mental disorder that affects roughly 1% of the world population (Saha, 2005). Schizophrenic patients report a combination of positive, psychotic symptoms including hallucinations and delusions in addition to several neurocognitive deficiencies (generally attributed as the negative and cognitive symptoms of schizophrenia.) These symptoms are outlined in the table below and there are key differences in the distribution of positive, negative, and cognitive symptoms when comparing schizophrenia patients to those of affective psychosis such as bipolar disorder and psychotic depression (Saha, 2007). While some schizophrenic subjects are more aware than others that their positive psychotic symptoms cannot be a manifestation of reality, the persistent hallucinations and delusions remain a relentless issue for the patient which can affect their ability to socially integrate in society; patients may also experience impaired judgment due to these positive effects; for example, a schizophrenic patient acting upon an auditory hallucination which he/she perceived to have heard, despite its absence in reality.

Table of Positive, Negative, and Cognitive Symptoms in Schizophrenia

Positive Effects	Negative Effects	Cognitive Effects
Hallucinations	Speech Poverty (Alogia)	Attention deficit
Delusions	Lack of emotion / interest /Asociality	Verbal, Visual, and Working Memory deficit
	Apathy or Inexpression	Executive Function (Reasoning) deficit
	Anhedonia	Social Cognition deficit

Table 1. List of Positive (Psychotic), Negative, and Cognitive effects of Schizophrenia

Although the more commonly mentioned symptoms of schizophrenia tend to be positive or psychotic in nature, the negative and cognitive effects of the disease can be equally debilitating. Negative symptoms, as implied in the name, represent an absence or reduction, for example, in speech (alogia). Anhedonia – an inability to feel pleasure – and blunted affect, characterized by reduced general expression, are also considered to be negative schizophrenic symptoms (Andreasen, 1982.) The cognitive symptoms of schizophrenia, as outlined by the MATRIX (Measurement and Treatment Research in Cognition in Schizophrenia) project, adversely affect “speed of processing, sustained attention, working memory, verbal learning, visual learning, executive function, and social cognition” (Nuechterlein, 2004). The cognitive changes associated with schizophrenia may present earlier than symptoms of psychosis and last the duration of the patient’s lifetime (Ibrahim, 2012).

Positive symptoms can be treated with typical and atypical antipsychotics as detailed later in the text. Many antipsychotic drugs do not eliminate the negative or cognitive symptoms of schizophrenia; however, these symptoms are often the reason schizophrenic patients have difficulty participating in society (for example: cognitive dysfunction resulting in inability to live independently, ineptitude in social situations making it tough to maintain relationships; lack of motivation/interest leading to diminished quality of life in general (Miyamoto, 2012).

The life span of an average schizophrenic subject is usually 15 years shorter than the general population, indicating additional costs associated with the disease that expand beyond the positive and negative symptoms described above (Saha *et al.*, 2007). The risk of morbidity is further exaggerated in schizophrenic patients displaying conditions such as respiratory and cardiovascular disease (Olfson, 2015). As with many mental disorders the risk of suicide is

increased and studies estimate that patients experience a lifetime risk of suicide around 5-10% (Hor, 2010; Freedman 2003), this risk, although still present, can be exponentially lowered with treatment, particularly with atypical antipsychotic clozapine, detailed later in this paper (Meltzer, 2013). Most patients experience an onset of the disease between ages 15-35 (Kurita *et al.*, 2016). Given the nature of the disease, it becomes apparent that schizophrenia is a mentally and physically debilitating condition with costs reaching beyond the initial symptoms displayed by patients.

Dopamine in Schizophrenia and the Emergence of Typical Antipsychotics

Schizophrenic symptoms are generally attributed to dysfunction in dopamine neurotransmission in the brain. Dysregulation of the dopamine circuit may present itself in schizophrenic patients as an overstimulated mesolimbic dopamine pathway with a concurrent lack of dopamine activity in the mesocortical pathway (Davis *et al.*, 1991). The positive symptoms of schizophrenia are associated with the hyper stimulation of neuronal projections on D2 receptors in the mesolimbic pathway. On the other hand, dopamine D1 receptors experience hypo-stimulation due to mesocortical projections to the prefrontal cortex being less active (Guillin, 2007).

These alterations produced by schizophrenia can affect the activity of pyramidal neurons in the prefrontal cortex, which serve roles in information processing, but also in the interneuron, which plays key regulatory roles in neurotransmitter release by acting on pyramidal cells. (Freedman, 2003.) The general function between these types of cells is shown to be disrupted by evidence suggesting that the density of synaptic connections between neurons and interneurons is

reduced. Activity between neurons and interneurons can be modulated by dopamine afferents but also by serotonergic, cholinergic, and noradrenergic cortical afferents (Freedman, 2003).

Before exploring these other neurotransmitters systems, a discussion of the first generational class of antipsychotic drugs focused on the dopamine system, is necessary. Specifically, the first antipsychotics focused on dopamine receptor antagonism due to the establishment of hyperexcitation in dopamine pathways leading to positive psychotic symptoms. This class of antipsychotics were known as first generational antipsychotic drugs and include haloperidol and perphenazine. While effective in mediating antipsychotic effects through D2 receptor blockade, this generation of antipsychotic drugs presented patients with side effects such as weight gain, temperature dysregulation, and most notably, extrapyramidal side effects (EPS) at clinically effective doses (Leucht, 2009). These EPS effects can be compared to the tremors and bradykinesia experienced by Parkinson's patients and result in overall reduction of motor function. A distinction between typical and atypical antipsychotics can be made by the absence of EPS in atypical antipsychotics (discussed further below); for this reason, many first generation APDs are classified as typical. Although, typical APDs seemed to be somewhat effective in alleviating positive symptoms, the presence of EPS symptoms and consideration of targets other than dopamine receptors encouraged research of a second generation of antipsychotic drugs.

Serotonin in Schizophrenia and the Emergence of Atypical Antipsychotics

As mentioned above, many other neurotransmitter systems are implicated in schizophrenia in addition to dopamine dysregulation. Unlike first generational APDs which were often supplemented by anti-parkinsonian drugs to limit their extrapyramidal side effects, patients

using second generation APDs reported fewer EPS symptoms. Clozapine was the first such drug developed (Meltzer, 2012), and its therapeutic effects were achieved by a combination of dopamine D1 and D2 antagonism coupled with 5-HT_{2A} antagonism.

Clozapine and other second generational APDs developed after it are generally considered atypical since they do not induce the EPS motor disturbances associated with administration of typical first generational APDs; however, there is some variation in the exact classification of typical and atypical APDs (Meltzer, 2013). Other atypical drugs which inhibit both D2 and 5-HT_{2A} receptors, in a manner similar to clozapine include risperidone and quetiapine (Tran *et al.*, 1997; Small *et al.*, 1997). Later atypical psychotics acting as full or partial agonists and antagonist in dopamine and serotonin receptors were also developed; this includes amisulpride (strong 5-HT₇ antagonist) and cariprazine (partial 5-HT_{2B}/5-HT_{1A} antagonist/agonist). All of these drugs are characteristic of positive symptom suppression.

In terms of efficacy, clozapine was shown to decrease positive psychotic symptoms with exceptional efficacy in patients displaying Treatment Resistant Schizophrenia (TRS) which accounts for roughly 30% of all schizophrenic patients (Meltzer, 1997); a patient is considered to have TRS following two or more unsuccessful treatments with other APDs. Although it is not clear whether atypical antipsychotics are superior to typical antipsychotics in terms of efficacy in non TRS patients, the presence of motor side effects (possibly resulting in the need to take additional drugs to limit these effects) in typical APDs may be a key factor in selecting atypical drugs over typical APDs.

The focus of many early antipsychotics in regulating solely dopamine receptor activity before the later development of multitargeted atypical antipsychotics to provide simultaneous

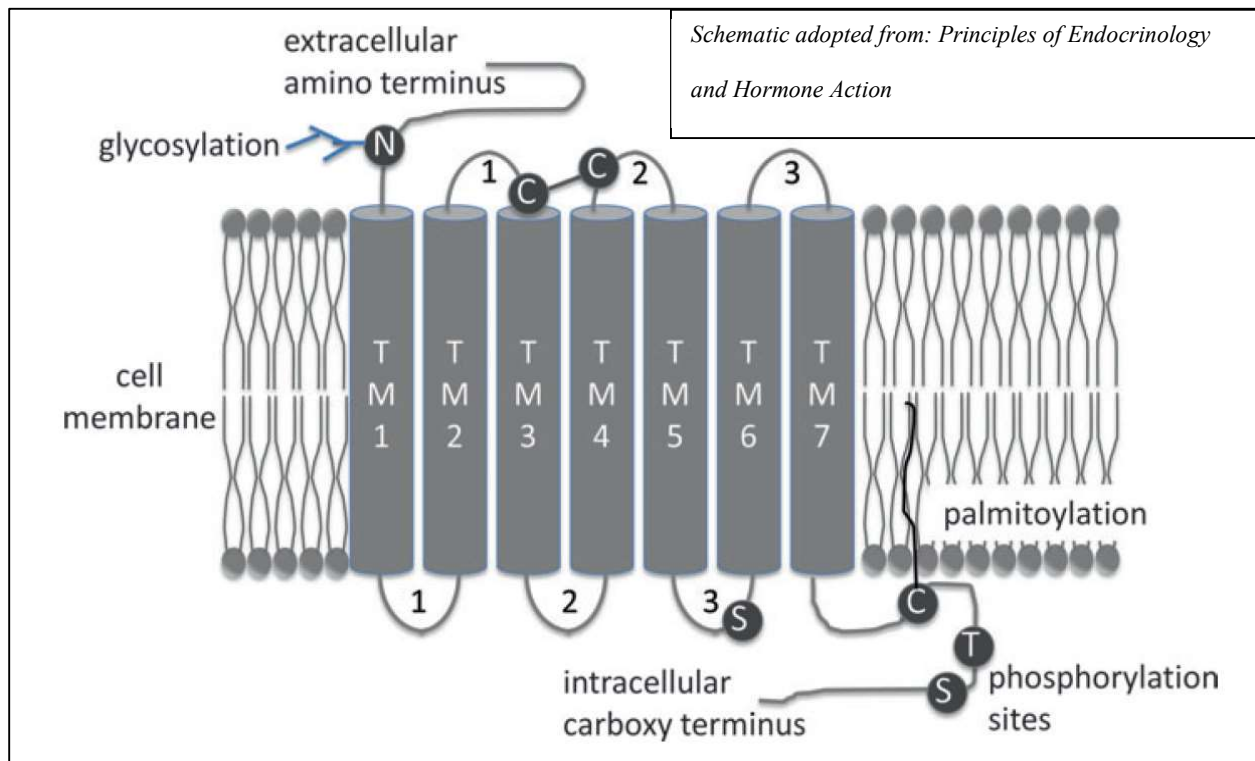
dopaminergic and serotonergic inhibition begs a question: what other neurotransmitters or receptors can be considered in the characterization of schizophrenia in addition to dopamine, serotonin, and their respective receptors? To assess this, we begin by considering psychedelics, which are intimately involved with the serotonin receptor 5-HT_{2A}.

General GPCR characteristics and Psychedelics as a Model of Schizophrenia

Psychedelic compounds can produce altered perception and distort feeling and include the compounds LSD (lysergic acid diethylamide) and psilocybin (C₁₂H₁₇N₂O₄P) (Hanks, 2016). LSD was first discovered by Dr. Albert Hoffman in 1943 after which the structural similarities between serotonin and LSD were examined more closely, and psychedelic substances were ultimately categorized as 5-HT_{2A} serotonin receptor agonists. Psychedelic substances induce feelings like those of positive schizophrenic symptoms (Hanks, 2016). Due to this, psychedelics have been used in numerous animal models of schizophrenia in which psychedelic agonists, but not non-psychedelic 5-HT_{2A} agonists, produce a head twitch response, a behavior analogous to positive hallucinogenic symptoms, in rodents (González-Maeso, 2007). The head twitch movements are not found in knockout mice missing the 5-HT_{2A} receptor (González-Maeso, 2007); thus, suggesting that the psychedelic effect of LSD and other psychedelics such as psilocybin is mediated by 5-HT_{2A} agonist activity.

The affinity of psychedelic compounds to GPCRs such as 5-HT_{2A} is of interest as the serotonin receptor, particularly the 5-HT_{2A}, is a target of atypical antipsychotics, such as clozapine detailed above. Since dopamine D1 and D2 receptors as well as the 5-HT_{2A} receptor fall under the classification of GPCRs, the general structure and functional basis of GPCRs should be considered.

Serotonin (5-HT), Dopamine (D1/2), and metabotropic Glutamate (mGlu2/3) receptors (detailed below) fall under the family of GPCRs of which over 800 different types were identified in the human genome (Davenport, 2013). GPCRs contain 7 conserved heptahelical transmembrane domains connected by intracellular and extracellular loop residues; an extracellular N terminus and intracellular C terminus is also characterized by sites for extracellular ligand binding and glycosylation, and intracellular phosphorylation (Gonzalez-Maeso, 2006). As the name suggests, GPCRs allow for intracellular signaling cascades in which ligand binding allows for activation of attached G protein; more specifically the alpha subunit of the GPCR dissociates from the beta/gamma subunits and allow the subunits to regulate respective effectors. The adopted schematic below from *Principles of Endocrinology and Hormone Action, 2018* shows the general features of a GPCR mentioned above.



Depending on the type of G protein, a signaling cascade involving activation of second messengers such as cAMP, calcium, and protein kinases can be detected (Pincas *et al.*, 2018). For example, the 5-HT_{2A} receptor is G_{q/11}-coupled resulting in Inositol trisphosphate and diacylglycerol (IP3/DAG) mediated calcium release from the endoplasmic reticulum upon receptor activation. The metabotropic glutamate receptor, discussed later in the paper, utilizes G_{i/o}-coupled signaling, characteristic of inhibiting adenylyl cyclase, and thus downstream cAMP production (Gonzalez-Maeso, 2011.) The dopamine D2 receptor is also G_{i/o}-coupled while the D1 receptor is G_{s/olf}-coupled and has essentially the opposite effect: activation of adenylyl cyclase and activation of cAMP (Gurevich, 2016). Finally, G_{11/12}-coupled GPCRs activate the Rho family of GTPases and serve functions primarily in cytoskeletal remodeling and migration of fibroblast and endothelial cells (Dhanasekaran, 1996; Shan 2006).

GPCRs were originally separated into 3 major classes based on structure: class A rhodopsin like receptors, class B secretin family receptor, and class C metabotropic glutamate receptors. However, adhesion class and frizzled class GPCRs can also be considered for a total of 5 families (Lee *et al.* 2015). There is no shared sequence homology among the different classes of GPCR; the greatest homology usually occurs in the cores of transmembrane domains of GPCRs within the same family. GPCRs contain orthostatic sites for endogenous ligand binding but also contain allosteric sites which do not bind ligand; rather they bind to Positive (PAMS) or Negative Allosteric Modifiers (NAMS) which can modulate response to an orthostatic agent (ligand) binding - the existence of several potential targets present in GPCRs may explain why almost 35% of FDA approved drugs target GPCRs (Foster & Conn, 2017; Hauser, 2018).

Classically, GPCRs were considered functional monomeric entities; this was based on evidence that purified monomeric family A GPCRs such as β 2-adrenoreceptor, Rhodopsin, and the μ -opiate receptor reconstituted in a phospholipid bilayer, were sufficient for activation (Whorton, 2007; Whorton, 2008; Kuszak, 2009). However, later evidence reported with regard to dopamine D2 receptors, that a minimal signaling unit of 2 receptors and one protein can cause activation, suggesting that class A receptors may homodimerize (Han *et al.*, 2009). The asymmetrical interface of the D2 protomers seemed to modulate each other upon agonist and inverse agonist binding further supporting the case for GPCR dimerization. The topic of Class A GPCR dimerization is still debated today; however, other GPCRs such as class C metabotropic Glutamate (mGlu) and GABA_B receptors have been reported to act as obligate dimers. For example, the mGlu family homodimers are linked covalently through disulfide bridges connecting their N-terminal Venus fly trap domain (Niswender, 2010.)

Studies involving the GABA_B (γ -aminobutyric acid type B) receptor also shed light on GPCR dimerization, specifically heterodimerization. GABA is the main inhibitory neurotransmitter acting through GABA_B receptors; the original gene for the receptor, GABA_B-R1 was cloned in 1997 but the receptor was not expressed in cell lines. However, one year later, the GABA_B-R2 subtype was shown to form a heteromeric complex with GABA_B-R1 as shown in several studies (Jones, 1998, White, 1998; Kaupmann 1998.) Alone, neither protomer can both bind ligand and activate the G protein, but co-expression of both the GABA_B-R1 and GABA_B-R2 lead to a fully functional GABA_B receptor (Jones, 1998, White, 1998; Kaupmann 1998).

Dimerization involving GPCRs of distinct subtypes/families include the κ and δ opiate receptors in which the protomers have been shown to heteromerize and modulate each other

(Jordan, 1999). Another such heteromeric complex including distinct families of GPCRs and is of especial interest in schizophrenia is the mGlu2/5-HT_{2A} heteromeric complex.

The mGlu2/5-HT_{2A} Heteromeric Receptor Complex

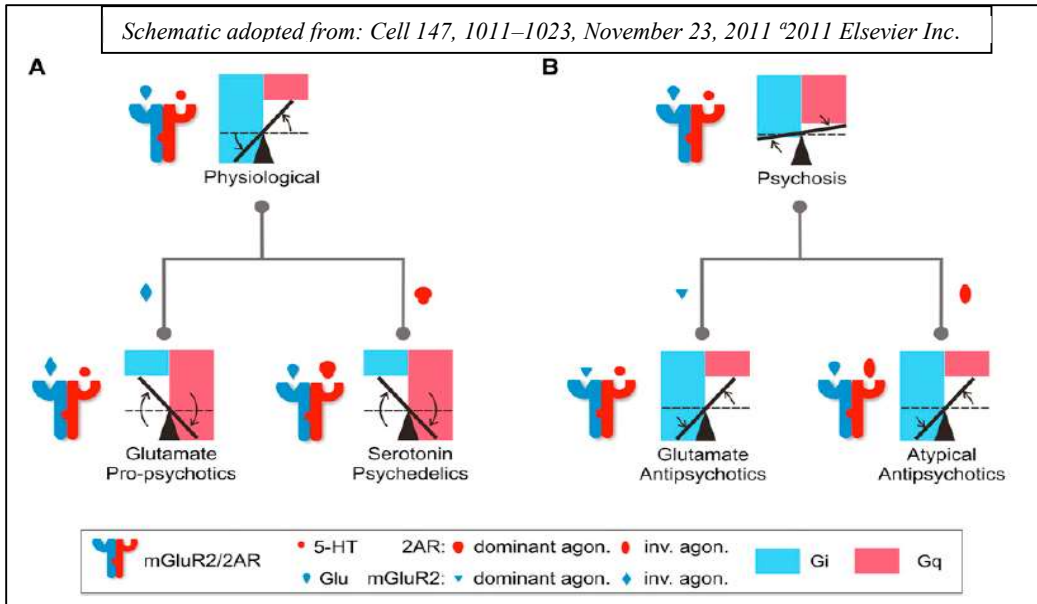
As mentioned previously, psychedelics such as LSD can stimulate the 5-HT_{2A} receptor resulting in psychosis like behavior, which can be behaviorally quantified in animal models. Recall that 5-HT antagonism is a mechanism of many second generational atypical antipsychotic drugs; thus, a GPCR containing 5-HT_{2A} complexed with a glutamate receptor (glutamate is a main excitatory neurotransmitter in the brain) is of interest when considering therapeutic drugs for schizophrenia. Additionally, mGlu2/3 has been a target for some therapeutic agonists such as LY2140023 which have shown improvement in the positive and negative symptoms of schizophrenia (Patil, 2007) However, other mGlu2/3 agonists such as LY379268 require 5-HT_{2A} expression inducing head twitch behavior in order to provide antipsychotic action in mice (Moreno, 2011; Hideshima, 2018).

Although initial postmortem brain studies of schizophrenic patients reported mixed results regarding the density of serotonin receptors, a 2008 study using binding assays found that cortical membranes of untreated schizophrenic patients had increased densities of 5-HT_{2A} receptors with concurrent decreases in mGlu2/3 levels. This increase in 5-HT_{2A} receptor density was not found in postpartum brains of patients treated with clozapine, which was attributed to chronic antipsychotic administration downregulating levels of 5-HT_{2A} (Gonzalez-Maeso, 2008). Furthermore, the mouse studies found that “the mGluR2 component of the [mglu2/5-HT_{2A}] complex eliminates the hallucinogen-specific component of the signaling in response to LSD-

like drug; [thus this pattern of 5-HT_{2A} and mGlu2 density may predispose schizophrenia.]”
(Gonzalez-Maeso, 2008.)

Functional cross talk has also been established between the two protomers of the 5-HT_{2A}-mGlu2 complex and is of relevance to schizophrenia. Recall the several pathways by which GPCRs can conduct a signal – the 5-HT_{2A} receptor is G_{q/11} coupled while the mGlu2 receptor is G_{i/o}-coupled. In general, the G_{i/o} coupled pathways inhibit the production of adenylyl cyclase while G_{q/11} pathways lead to an increase in calcium release mediated by IP3/DAG. The existence of multiple signal transduction pathways in the heterodimer allows for assessment of the downstream interactions induced by either protomer. For example, researchers found that when the 5-HT_{2A}-mGlu2 complex was stimulated with LY379268, an mGlu2 agonist, there was also activation of G_{q/11} proteins by the 5-HT_{2A} portion of the complex; this did not occur in 5-HT_{2A} deficient mice (Moreno, 2016).

Further studies on the mGlu2/5-HT_{2A} heteromeric complex, suggest that in response to endogenous ligands glutamate and serotonin, the mGlu2/5-HT_{2A} heteromer establishes a G_{i/o}-G_{q/11} balance which may result in behavioral responses different from 5-HT_{2A} and mGlu2 homomers (Fribourg, 2011). In summary, the assembly of the heteromeric complex reduces 5-HT mediated G_{q/11} signaling and increases mGlu2 mediated G_{i/o} signaling. There is a G_{i/o}-G_{q/11} imbalance in psychosis that can be modulated by 5-HT_{2A} and mGlu2 agonists or inverse agonists. For example, 5-HT agonists and mGlu2 antagonists shift the G_{i/o}-G_{q/11} balance to those more similar to psychotic conditions than physiological conditions. Likewise, mGlu2 agonists and 5-HT inverse agonists would accelerate G_{i/o} signaling while diminishing G_{q/11} signaling – shifting the balance towards physiological conditions. See adopted schematic:



In a condition as complicated as schizophrenia, with several interconnected brain pathways being in balance with one another, there is a need for drugs that are multi-targeted. The transition from typical to atypical antipsychotic drugs shows the importance of considering additional protein targets involved in schizophrenia. The 5-HT_{2A}-mGlu2 receptor complex is a prime example of such targets.

Mutational Approaches to Map the Heteromeric Interface of 5-HT_{2A}-mGlu2

Several studies have taken a mutation-based approach to assess the hetero-dimeric interface of the mGlu2-5-HT_{2A} complex. A study using immunocytochemistry verified the colocalization of 5-HT_{2A} and mGlu2 in tissue. These studies shed light on the phenomena that mGlu2 but not mGlu3 colocalizes in cortical tissue and in cell culture (Gonzalez-Maeso, 2008.) A similar discrimination between 5-HT_{2A} and 5-HT_{2C} (in which only 5-HT_{2A} but not 5-HT_{2C} can dimerize with mGlu2) is used to assess the aims of my study.

Specifically, previous studies have demonstrated the importance of TM4 in mGlu2 in mediating the heteromeric interface of the mGlu2-5-HT_{2A} heterocomplex (Moreno, 2012). This study, in which residues of mGlu3 are swapped into mGlu2 at the predicted sites within the heteromeric interface, was assessed by coimmunoprecipitation to see if the interface could be broken. By this method, researchers were able to identify TM4 but not TM5 of mGlu2 as necessary for 5-HT_{2A}-mGlu2 heteromerization (Moreno, 2012). Furthermore, the researchers created mutant mGlu3 chimeras with a TM4 domain from mGlu2 and found upon immunoprecipitation that TM4 of mGlu2 was also sufficient for mediating the heteromeric interface. (Moreno, 2012)

A follow up study also implicated TM4 of 5-HT_{2A} to be involved in mediating the heteromeric interface of the mGlu2-5-HT_{2A}. This study first confirmed that WT 5-HT_{2A} but not WT 5-HT_{2C} coimmunoprecipitated with mGlu2 and could form a complex (Moreno, 2016.) As mentioned earlier, this relationship between 5-HT_{2A} and 5-HT_{2C} is analogous to that of mGlu2 and mGlu3. The mutant chimeras created in my thesis experiments included aligned residues from TM4 of 5-HT_{2C} within portions of the TM4 of 5-HT_{2A} in attempt to disrupt the binding interface of the heteromer.

After Identifying TM4 of mGlu2 as the domain responsible for mediating heteromerization. Researchers identified Ala-677^{4.40}, Ala-681^{4.44}, and Ala-685^{4.48} as the individual amino acids responsible for the heteromeric interface in mGlu2 (Moreno 2012). The numbering system used to describe the residues above follows the Ballesteros-Weinstein numbering system for conserved GPCR residues (Ballesteros, 1995); in this system, the most conserved residue across species, R, within a given transmembrane domain, X, is denoted as

R_{X.50}. All residues adjacent to R_{X.50} towards the N terminus are given sequentially decreasing numbers (R_{X.49}, R_{X.48}, etc.) while those towards the C terminus are given sequentially increasing numbers relative to R_{X.50}). Since the *Moreno, 2012* experiment was concerned with residues in TM4 of mGlu2, we see the corresponding Ballesteros-Weinstein numbering used. The findings of the paper were based on substitutions at these positions resulting in the abolishment of hallucinogenic behavior. A follow up study, with an emphasis on identifying a direct interaction between these residues and the heteromeric interface, utilized amber codon suppression to introduce photoreactive unnatural amino acid azF (p-azido-L-phenylalanine) at these sites along the intracellular end of TM4; they concluded that Ala-677^{4.40}, Ala-681^{4.44}, and Ala-685^{4.48} of mGlu2 are directly involved in mediating the heteromeric interface (Maeso Group, unpublished).

While this thesis is primarily focused on identifying halves of the transmembrane domain responsible for heteromerization rather than individual amino acids, the greater goal of this project is to eventually identify the individual residues of 5-HT_{2A} responsible for mediating the heteromeric interface by following a similar approach taken with mGlu2.

Coimmunoprecipitation studies can identify whether portions of 5-HT_{2A} TM4 are involved in heteromerization but a coimmunoprecipitation assay cannot establish a direct interaction between amino acids. Several approaches, however, have described the use of photoreactive unnatural amino acids in GPCRs to cross link individual residues in close proximity with one another; such studies have been conducted in the CXCR4 GPCR implicated in HIV, and the neurokinin-1 (NK1) family A GPCR (Grunbeck, 2011; Valentin-Hansen, 2014). The general method for this approach involves co-transfection of a “cocktail” of plasmids – a synthetase gene, a suppressor tRNA gene, and a gene encoding the GPCR to introduce an unnatural amino acid (UAA) at a site of interest, such as azF; when excited by ultraviolet light, azF can form chemical cross links with

a sensitivity of 3-5 angstroms; indicating the presence or lack of a direct interaction by the residues mutated (Huber, 2014). The mutant constructs created in this thesis, and the interpretation of their Co-IP results, can be used as a starting point in identifying individual amino acids by the photo-crosslinking methods described above, if found to be implicated in mediating the heteromeric interface of the mGlu2-5HT_{2A} complex.

The dysregulation of several neurotransmitter systems in schizophrenia warrants closer consideration of the GPCRs involved in each pathway. The limitations of early antipsychotics focused primarily on dopamine receptors to alleviate the positive symptoms of schizophrenia were addressed by assessing the roles of other GPCRs such as 5-HT_{2A} in the production of atypical antipsychotics. The benefit of such exploration demands consideration of additional proteins and protein complexes implicated in schizophrenia, such as the 5-HT_{2A}-mGlu2. We consider a mutation-based approach to identify which portion of the 5-HT_{2A} is involved in mediating the heteromeric complex.

Objectives

Due to the biological relevance of GPCRS in neuropsychiatric disorders generally, and keeping in mind the changes in receptor density, particularly of the mGlu2 and 5-HT_{2A} GPCRS in schizophrenia and their colocalization, this study aims to identify a portion of TM4 of the 5-HT_{2A} responsible for mediating the heteromeric interface within the mGlu2-5-HT_{2A} complex.

The implication of transmembrane domain four (TM4) within the 5-HT_{2A} receptor in mediating heteromerization of the mGlu2-5-HT_{2A} complex allows deeper investigation into the residues contained within it. It also begs a question: which half of the transmembrane domain may contain the residues responsible for the interface. With this in mind, my Masters research aims to identify whether the residues in the N terminal half of TM4 of 5-HT_{2A} are involved in heteromeric complex formation. Because research has established that TM4 of 5-HT_{2A} is necessary to form the mGlu2-5-HT_{2A} complex (Moreno et al 2016), the study aims to ultimately identify residues within the TM4 of 5-HT_{2A} that are necessary to form the mGlu2-5-HT_{2A} complex. To do this, I substituted residues in half of the TM4 of 5-HT_{2A} closest to the N terminus, with aligned residues in 5-HT_{2C} to create 2 constructs. One of these constructs is considered partially mutated (5-HT_{2A}-TM4ΔNP) while the other is considered a full mutation of half the TM4 residues lying closest to the N terminus (5-HT_{2A}-TM4ΔNF). Essentially, the 5-HT_{2A}-TM4ΔNP (partial) chimera contains swapped 5-HT_{2C} residues spanning a quarter length of the TM4 closest to the N terminus, while the 5-HT_{2A}-TM4ΔNF (full) construct is mutated to include residues from 5-HT_{2C} along the entire half of the TM4 of 5-HT_{2A} closest to the N terminus. All three constructs, (WT 5-HT_{2A}, 5-HT_{2A}-TM4ΔNP, 5-HT_{2A}-TM4ΔNF) were transfected individually or co-transfected with mGlu2 and prepared into membranes before

running a co-immunoprecipitation experiment and subsequent western blot to test whether complex formation is disrupted. If the N terminal half of TM4 does indeed include residues responsible for mediating complex formation, we would expect a stronger Co-IP signal from the sample containing WT mGlu2 & 5-HT_{2A} than compared to one containing co-transfection of mGlu2 & the Mutant 5-HT_{2A}-TM4ΔNF or 5-HT_{2A}-TM4ΔNP. Furthermore, we would expect the lack of a band (or at least a less intense band) in the coimmunoprecipitation of mGlu2 and any mutant chimera originally containing the residues responsible for mediating the heteromeric interface. This information would be extremely valuable in future studies pertaining to the greater goals of this project; the possibility of using photo-crosslinked unnatural amino acids would allow identification of individual 5HT_{2A} residues directly interacting with the heteromeric interface of the mGlu2-5HT_{2A} heteromeric complex.

Materials & Methods

Wild Type Plasmid Preparation

The constructs pcDNA3.1-c-Myc-5-HT_{2A}-mCherry and pcDNA3.1-HA-mGluR2-mCitrine, described previously in Moreno et al., 2016, were obtained. In brief, the WT metabotropic glutamate receptor was tagged N terminally with hemagglutinin (HA) and C-terminally by mCitrine (a type of yellow fluorescent protein.) The WT serotonin receptor 5-HT_{2A}, alternatively, is N-terminally tagged by c-Myc and C-terminally tagged with mCherry (a type of monomeric red fluorescent protein.) All mutant 5-HT receptor chimeras include the same tags described. Tagging receptor constructs allows for use of antibodies specific to the tag – and thus the construct – during immunoblot and immunoprecipitation assays. Plasmids were purified and amplified by methods detailed below.

Chimeric Human 5-HT_{2A} Constructs with half of TM4 near N terminus Mutated with residues of 5-HT_{2C}: partial (5-HT_{2A}-TM4ΔNP) and full (5-HT_{2A}-TM4ΔNF)

In accordance with manufacturer instruction, QuickChange II site-directed mutagenesis kit (Stratagene) was used to modify the primary structure of the Human 5-HT_{2A} receptor into full and partial chimeric constructs containing residues of 5-HT_{2C} in the N terminal half of TM4. The protocol involves the designing of primers before adding them to template DNA and cycling temperatures to induce the mutations desired. For the partial mutant 5-HT_{2A}-TM4ΔNP, the following forward and reverse primer were designed (duplex forming nucleotides highlighted):
Forward: 5'-

ATGGTCCAAACAGCAATGATTTTCATTGATTGCCTTAGTTCTGGAGTTGAAGC-3'.

Reverse: 5'-

GCTTCAACTCCAGAACTAAGGCAATCATGTGAAAATCATTGCTGTTTGGACCAT-3'

The primers along with appropriate manufacturer included buffers and template c-myc-5-HT_{2A}- m-cherry (WT) construct DNA were Thermo-cycled with the following parameters: 1 cycle at 95°C followed by 16 cycles of 30 minutes at 95°C, 1 minute at 55°C and 8 minutes at 68°C. Following this, a restriction enzyme Dpn1 was used for 1 hour at 37°C to digest parent amplification products to yield the partial 5-HT_{2A}-TM4ΔNP construct. This chimera (TM4ΔNP) had the effect of substituting the consecutive Phenylalanine and Leucine found in WT 5-HT_{2A} with the consecutive Isoleucine and Methionine found in WT 5-HT_{2C} (Fig 3 & 4).

For the construct (5-HT_{2A}-TM4ΔNF) involving a complete mutation of the residues that lay in the half of TM4 closest to the N terminal of 5-HT_{2A}, a similar approach to creating the partial chimera was used; however, the DNA template used before cycling was the 5TM4ΔNP partial construct described above. The forward and reverse primers designed for the mutation as follows (duplex forming nucleotides highlighted):

Forward: 5'-

GATATACCTACTGATATGGTCCAAACAATAGCGATTTTCATGATTGCCTTAGTTCTG
GAG-3'

Reverse: 5'-

TCCAGAACTAAGGCAATCATGAAAATCGCTATTGTTTGGACCATATCAGTAGGTATA
TC-3'

The parameters for thermocycling were as follows: 1 cycle at 95°C followed by 18 cycles of 30 minutes at 95°C, 1 minute at 55°C and 7 minutes at 68°C. Similar to the partial construct, Dpn1 restriction enzyme was used to digest the unmutated parent DNA. Ultimately this produced the full N terminal half TM4 chimera 5-HT_{2A}-TM4ΔNF by substituting the consecutive Isoleucine and Alanine found in the 5-HT_{2A}-TM4ΔNP (and in WT 5-HT_{2A}) with the consecutive Alanine and Isoleucine found in 5-HT_{2C} respectively. (Fig 3 & 4).

Since all other residues located on the half of TM4 closest to the N terminal end of the GPCR are the same when considering the aligned sequence of 5-HT_{2A} and 5-HT_{2C}, the above two constructs are sufficient to assess the hypothesis of the paper.

Purification, Amplification, and Sequencing of Constructs

Wild Type constructs obtained were transformed into XL1-Blue super competent cells using the heat shock method and let to incubate overnight on Agar plates, before a single colony was picked. This colony was let to grow on a shaker in LB broth at 37°C overnight before purification and amplification by the protocol described in QIAGEN Plasmid Maxi Prep.

The products from the Site Directed Mutagenesis were transformed in a similar manner described above; however, the selected colony was first amplified using QIAGEN Plasmid Mini prep before being mixed with primer to be sent for sequencing. The primer used for sequencing of both Mutant constructs (5-HT_{2A}-TM4ΔNP & 5-HT_{2A}-TM4ΔNF) was as follows.: 5'-AGC TGA TAT GCT GCT GGG TT -3'. Following sequence verification, the constructs were retransformed and plated overnight, a colony to grow in broth was selected, and a Maxi Prep to amplify yield of the construct was conducted.

Maintenance of HEK 293 Cells

Human Embryonic Kidney (HEK293) cells were used for transfection. These cells were incubated in a cell culture environment with 5% humidified CO₂ at 37°C. The media selected to maintain the cells, Dulbecco's modified Eagle's medium (DMEM), includes pH sensitive Phenol red and was further modified by the addition of dialyzed fetal bovine serum (dFBS) equal to 10% of the total volume of media and Penstrep equal to 1% of the total media volume (except immediately prior to transfection, as noted below). Cells were split routinely using fresh media in a ratio between 1:12 and 1:8, to allow for growth and for fresh media, and never passaged higher than 22 times.

Transfection of HEK 293 cells

Plates containing HEK 293 cells prepared for transfection or co-transfection were aspirated of any media and replaced with DMEM containing only 10% dFBS but no Penstrep prior to transfection (although reducing the chances of contamination, Penstrep inhibits transfection.) Plasmid containing the desired construct was added to a vial of Gibco Opti-MEM media before Invitrogen Lipofectamine 2000 reagent was added according to manufactures instructions in a 1:2 protein to lipofectamine ratio in a separate vial of Gibco Opti-MEM media; the mixtures were allowed to incubate separately for 1 hour. Following incubation, the mixtures were combined and added to Penstrep free plates containing HEK 293 cells in DMEM (+10% dFBS). Cells were grown to 80% confluency before transfection. Media containing lipofectamine was removed 24 hours following transfection; cells were allowed a maximum of 48 hours after transfection before harvesting as described below.

Using the method described above, transfections of single plasmids were done using 6µg of DNA per plate (Ex:8 plates = 48µg of plasmids total) This corresponds to a lipofectamine ratio of 12µg per plate or 96µg total. This includes the individual transfection of the constructs HA-mGluR2-mCitrine, c-Myc-5-HT_{2A}-mCherry, and c-Myc-5-HT_{2A}-TM4ΔNP-mCherry.

Considering co-transfection of the serotonin and glutamate GPCRs, the HA-mGluR2-mCitrine plasmid and c-Myc-5-HT_{2A}-mCherry plasmid were transfected in a ratio of 2:1 (For example, 6µg of mGlu2 and 3µg of 5-HT_{2A} for a total of 9µg DNA per plate to be combined with 18µg lipofectamine total). Co-transfection of the partial mutant c-Myc-5-HT_{2A}-TM4ΔNP-mCherry with HA-mGluR2-mCitrine was performed using the same ratio. The full mutant c-Myc-5-HT_{2A}-TM4ΔNF-mCherry is yet to be transfected.

Preparation of Membrane from Cell Pellets

48 Hours following transfection, cells were harvested using calcium and magnesium free DPBS (Dulbecco's Phosphate Buffer Saline;) the buffer allows cells to dissociate from the plate and be scraped into a falcon before centrifugation. The resulting cell pellets were frozen at -80°C before being prepared into membranes.

For such preparations, cell pellets were resuspended in Tris-HCL buffer and homogenized before centrifugation. Cell membrane isolation begins with Initial low speed centrifugation of the homogenized solution at 1,000 xg in 4°C, which pellets heavier cell organelles such as the nucleus. Centrifugation of the supernatant from the initial spin at high speed (40,000 xg) results in a cell membrane precipitate which can be washed with Tris-HCL and recentrifuged at the same speed. The resulting supernatant is thrown off leaving isolated cell membranes containing the GPCR plasmids of interest in the precipitate.

Co-Immunoprecipitation Studies

The initial co-immunoprecipitation was conducted using membrane preparations of co-transfected wild type HA-mGluR2-mCitrine and c-Myc-5-HT2A-mCherry containing 1mg (1000 μ g) of protein. In this assay the prepared membrane samples were first exposed to RIPA lysis buffer and then solubilized to allow the GPCRs within the membrane to become surrounded by surfactant before being rotated with Protein A/G PLUS-Agarose immunoprecipitation beads. For wildtype assays, anti-c-myc antibody (Cell Signaling, catalog #2276) in a 1:100 concentration was added to the beads mixture before overnight rotation at 4°C., Total Protein controls were taken prior to antibody addition and immediately after overnight rotation (supernatant from 14000RPM spin) before final solubilization of the immune complexes formed by the assay.

Products of the Co-IP (precipitate from 14000RPM spin after overnight rotation) were washed and resolubilized with buffer before being prepared for immunoblot analysis. An aliquot of the final wash step was also reserved for Western Blot analysis to detect Co-IP signal that may have been lost to the final wash steps.

Protein Estimation and Western blotting

In order to resolve equal amounts of protein in each lane during the SDS-PAGE process, the protein composition of membrane preparations was estimated using the Bradford Protocol. (Note that for co-transfected samples used in Co-IP experiments, protein composition was estimated prior to the Co-IP.) A spectrophotometer set to a wavelength of 595nm detects the blue-dye within reagent when bound to proteins. A standard curve was established by detecting the absorbance of increasing control amounts of Bovine Serum Albumin (BSA). The curve was

used to interpolate an estimated protein composition (in $\mu\text{g}/\mu\text{l}$) of the membrane preparations from cells transfected with WT c-myc-5-HT_{2A} and c-myc-5-HT_{2A}-TM4 Δ NP receptors.

Two immunoblot assays, one with the aim of analyzing the results of the Co-IP experiment (Figure 3) and another blot to verify expression of the partial chimera 5-HT_{2A}-TM4 Δ NP (Figure 4) were conducted and shown in the Results & Discussion section.

Western blot following Co-Immunoprecipitation:

All samples were heated to 95 degrees at 5 minutes before loading onto a 12% polyacrylamide gel. Lanes 1 and 2 with Total Protein and Total Protein Analog (supernatant described above) respectively, were loaded with 20 μl of sample and 5 μl of Laemmli buffer each. Lane 3, composed of sample from a late wash step, and Lane 4 containing the final Co-IP products were loaded with the same specifications. The samples were let to resolve at 60V for 1 hour and then 100V for 3 hours before overnight transfer to a nitrocellulose membrane in the cold room (30V). The membrane was blocked with buffer containing BSA, milk, and TBST to avoid nonspecific binding before adding antibody. The membrane was incubated in a 1:1000 dilution of primary antibody, HA-Rabbit Ab (Cell Signaling Catalog #2367), for 1 hour at room temperature. The membrane was then washed, blocked, and incubated in secondary antibody, Anti-Rabbit Horseradish peroxidase (HRP) conjugated Ab (Cell Signaling Catalog #7074) for 2 hours (1:5000 dilution) at room temperature before being visualized with enhanced chemiluminescence reagents (ThermoScientific) in accordance with manufacture instructions.

Western Blotting to Verify Expression of the Partial Chimera 5-HT_{2A}-TM4 Δ NP

All samples were heated to 95 degrees for 5 minutes before loading onto a 12% polyacrylamide gel. Both lanes of the gel were loaded with a volume equal to 20 μg of protein by mixing the

appropriate volume with 5 μ l of 5x Laemmli buffer and Tris-HCL up to a total sample volume of 25 μ l per lane. SDS-PAGE was conducted at 60V for 1 hour and 100V for 2.5 hours before being transferred onto a nitrocellulose membrane overnight at 30V in 4°C. Following wash and blocking steps, the membrane was incubated in primary antibody, c-Myc-Tag-Mouse Ab (Cell Signaling, catalog #2276) for 1 hour (1:000 dilution) before being washed, blocked, and incubated in Anti-Mouse Horseradish peroxidase (HRP) conjugated secondary Ab (Cell Signaling, catalog #7076) in a 1:5000 dilution. Visualization of the immunoblot of the chimeric construct was conducted using the same chemiluminescence reagents mentioned earlier.

Results and Discussion

Prior studies suggested that TM4 of 5-HT_{2A} is necessary for mediating heteromerization, with this in mind, my study developed two mutant constructs 5-HT_{2A}- Δ TM4NP and 5-HT_{2A}- Δ TM4NF, consisting of residues from 5-HT_{2C} swapped into sections of the TM4 domain. Although the chimeras are named “partial” and “full”, it is important to consider that they only consider one half, (the N terminal half,) of TM4 with regards to nomenclature. The sequencing results of both constructs are displayed in Figures 1 (next page) and suggest that the desired mutations were made in the correct positions. Figure 1 highlights which portions of TM4 are considered the N terminal half as opposed to the C terminal half.

The choice to design chimeric constructs mutated on the N terminal half of TM4 rather than on the C terminal half was simply a matter of convenience. Figure 1 and 2 (displayed on horizontal pages blow) clearly show the mutation of 2 sequential amino acids (FL \rightarrow IM) followed by the mutation of 2 other sequential amino acids (IA \rightarrow AI); because the mutated residues were sequential, they could each be created using a single site directed mutagenesis protocol. Figure 2 also highlights the individual nucleotides which needed to be swapped for the mutation to occur – notice in the orange box for example showing the partial chimeric construct (FL \rightarrow IM) only three out of the six nucleotides required to form the two residues needed to be swapped, while in the full TM4 chimera (IA \rightarrow AI) four out of 6 nucleotides needed to be changed. To simplify the process of making mutations, I chose to begin with the N terminal half of TM4. However, additional C terminal chimeras may need to be made in future studies, using the same strategies shown in figures 1 and 2, if the full N terminal half TM4 chimera (5-HT_{2A}- Δ TM4NF) fails to break immunoprecipitation of the 5-HT_{2A}-mGlu2 complex.

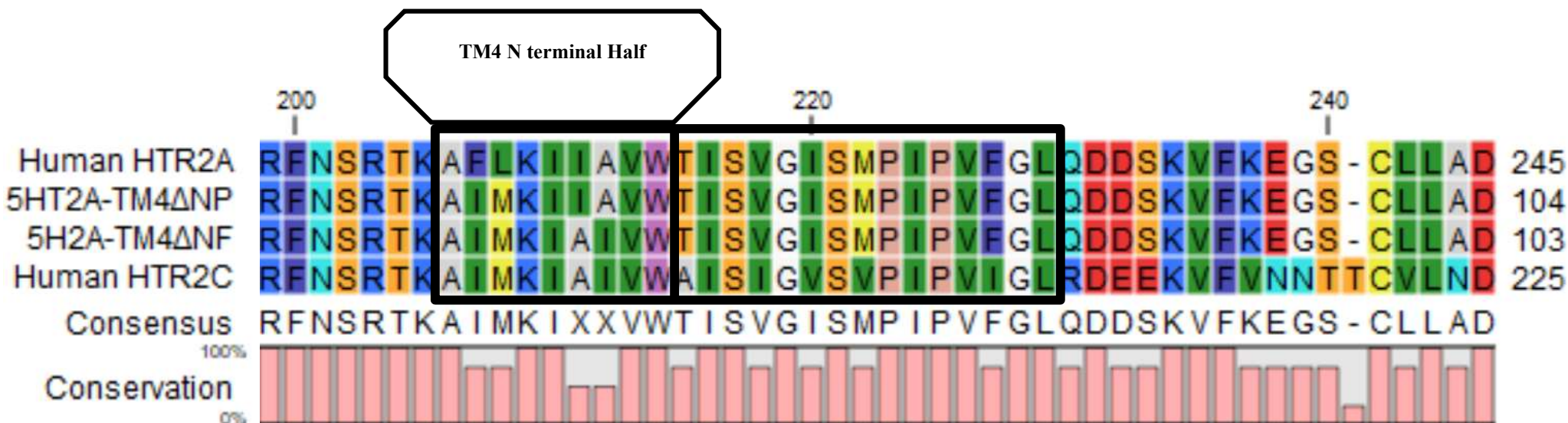


Figure 1. Alignment of residues in Human 5-HT_{2A} and 5-HT_{2C} along with both partial (5-HT_{2A}-TM4ΔNP) and full (5-HT_{2A}-TM4ΔNF) TM4 N terminal half mutants. The entire TM4 of 5-HT_{2A} receptor is outlined in black. The N terminal half of the Transmembrane domain is highlighted as the left box within the TM4 outline corresponding to A206_{4.42} through W214_{4.50}. Sequence consensus of all four constructs is displayed. The 5-HT_{2A}-TM4ΔNP construct replaces the phenylalanine_{4.43} and leucine_{4.44} found in 5-HT_{2A} with the consecutive isoleucine_{4.44} and methionine_{4.55} found in WT 5-HT_{2C}. The 5-HT_{2A}-TM4ΔNF construct includes the same residue changes as 5-HT_{2A}-TM4ΔNP with the following additions: the consecutive isoleucine_{4.47} and alanine_{4.48} found in 5-HT_{2A} are substituted with the consecutive alanine_{4.47} and isoleucine_{4.48} found in 5-HT_{2C}. Notice that all other residues denoted as within the TM4 N terminal half of 5-HT_{2A} share homology with the 5-HT_{2C} receptor so have not been mutated.

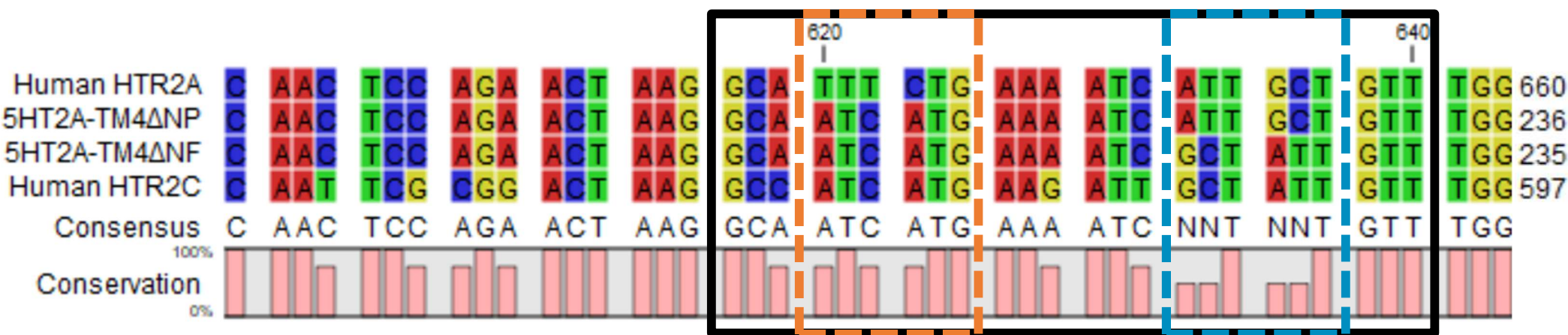


Figure 2. Nucleotide Sequence alignment of Human 5-HT_{2A} and 5-HT_{2C} and both partial (5-HT_{2A}-TM4ΔNP) and full (5-HT_{2A}-TM4ΔNF) TM4 N terminal half mutants. The black box denotes the half of TM4 closest to the N terminal of the GPCR. In the partial (5-HT_{2A}-TM4ΔNP) chimera, the TTT coding for Phenylalanine_{4.43} and CTG coding for Leucine_{4.44} from 5-HT_{2A} were replaced with ATC (Isoleucine_{4.43}) and ATG (Methionine_{4.44}) naturally found in 5-HT_{2C} as shown in the orange box. The blue box highlights changes made to produce the full (5-HT_{2A}-TM4ΔNF) chimeric construct in addition to the residues mutated in 5-HT_{2A}-TM4ΔNP: the ATT and GCT coding for Isoleucine_{4.47} and Alanine_{4.48} respectively were substituted with GCT and ATT, matching the sequence of 5-HT_{2C}. Notice that some nucleotides in the figure appear to differ in mutant constructs than in 5-HT_{2C}; it is important to note however that multiple codons can code for the same amino acids (ex: GCA and GCC both code for Alanine while ATC and ATT both code for Isoleucine). Thus, certain individual nitrogenous bases need not be mutated to achieve the sequence of residues desired for each construct

The coimmunoprecipitation experiment in this study was assessed by Western Blot shown in Figure 3 and confirms results obtained in (Moreno 2012) suggesting that WT 5-HT_{2A} and mGlu2 immunoprecipitated when co-transfected in HEK293 cells.

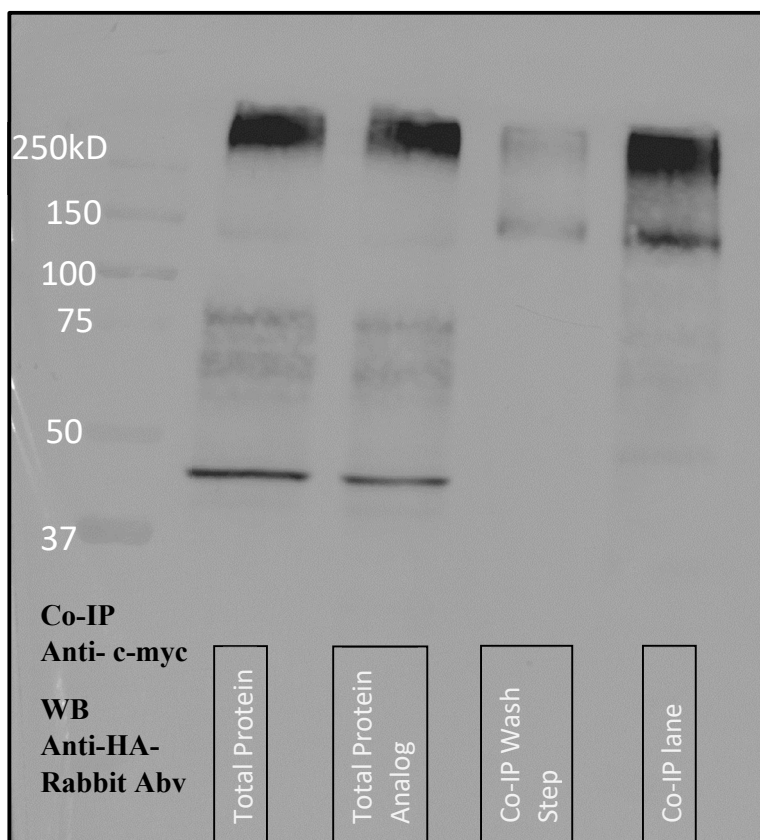


Figure 3. Western blot probing for HA (tagged as HA-mglu2) following Coimmunoprecipitation involving a c-Myc Antibody (tagged as c-Myc-5-HT_{2A}). Aliquots of sample before Co-IP antibody was added (lane 1) and samples of supernatant after overnight Co-IP rotation but before final wash or solubilization buffers were added (lane 2). Lane 1 and 2 are used as Total Protein references. Lane 4 corresponds to sample yielded after completing the entire Co-IP protocol, while Lane 3 includes sample from a late wash step which some Co-IP signal may have been lost to. All four lanes probed for HA-mGlu2 with Primary Antibody: HA-Tag Rabbit Ab; Secondary Antibody: Anti-Rabbit HRP Ab.

Figure 3 shows that the Co-IP lane (lane 4) has 2 clear molecular weight bands at near 120 kDa and near 240 kDa which correspond to WT HA-mGluR2-mCitrine in its monomeric and dimeric forms respectively (Maeso Group, unpublished). The wash step sample in lane 3 contains identical bands to those in lane 4; this is not surprising as the wash step sample was only included to detect any Co-IP signal lost to the final wash steps in the protocol. Lane 1 and 2 are total protein control lanes, meaning that signal in the Co-IP lane should not be present unless also present in the Total Protein lanes 1 and 2; our results are congruent with this idea. Bands displayed below 100 in the total protein lanes may be attributed to nonspecific binding which was lost in the Co-IP and wash lanes after final solubilization steps.

To assess the hypothesis of the paper, the full chimeric construct (5-HT_{2A}-ΔTM4NF) should first be coimmunoprecipitated with WT mGlu2. We can either expect the presence of a Co-IP band or absence of a Co-IP band

If 5-HT_{2A}-ΔTM4NF CAN coimmunoprecipitate with mGlu2 (band present)

There are multiple reasons for a result in which 5-HT_{2A}-ΔTM4NF and mGlu2 can coimmunoprecipitate and display a band. It may be that N terminal half TM4 residues do not mediate the heteromeric interface of 5-HT_{2A}-mGlu2 complex. Or they may still show Co-IP signal, but one that is weak in comparison to that of a full C terminal chimera which does not disrupt coimmunoprecipitation at all. The best way to assess this result would be to create a 5-HT_{2A} chimera considering the full C terminus half of TM4 (for example: 5-HT_{2A}-ΔTM4 $\underline{\text{C}}$ F) and compare the coimmunoprecipitation results from both constructs. If the 5-HT_{2A}-ΔTM4NF produces a band while the 5-HT_{2A}-ΔTM4 $\underline{\text{C}}$ F does not, then the residues responsible for mediating heteromerization are located in the C terminal half of the TM4 in 5-HT_{2A}. However, if

both constructs display a Co-IP signal, a quantification of the Co-IP signal is necessary. A method similar to *Moreno, 2012* in which coimmunoprecipitation of both N terminal and C terminal half TM4 chimeric constructs of mGlu2 containing residues from mGlu3 both produced Co-IP signal, can be followed: after running multiple experiments, the signals were aggregated and quantified in terms of immunoreactivity. The same approach can be used with multiple chimeras producing Co-IP signal. The chimera with the lowest immunoreactivity is likely to be responsible for mediating the heteromeric interface of 5-HT_{2A}.

If 5-HT_{2A}-ΔTM4NF can NOT coimmunoprecipitate (band absent):

This would validate the hypothesis of the experiment. A lack of Co-IP signal would indicate that the heteromeric interface was broken when the N terminal half was mutated – suggesting that one or more of original residues (phenylalanine^{4.43}; leucine^{4.44}; alanine^{4.47}; isoleucine^{4.48}) in the N terminal half of TM4 in 5-HT_{2A} mediate complex formation. Co-IP of the partial mutant could be used to assess which set of residues FL or IA is more responsible; however, a Co-IP experiment does not differentiate direct interaction of proteins from indirect interaction via additional scaffolding proteins. Additional approaches such as crosslinking photoreactive unnatural amino acids to identify the individual residues responsible, as described further in the future directions section can be used,

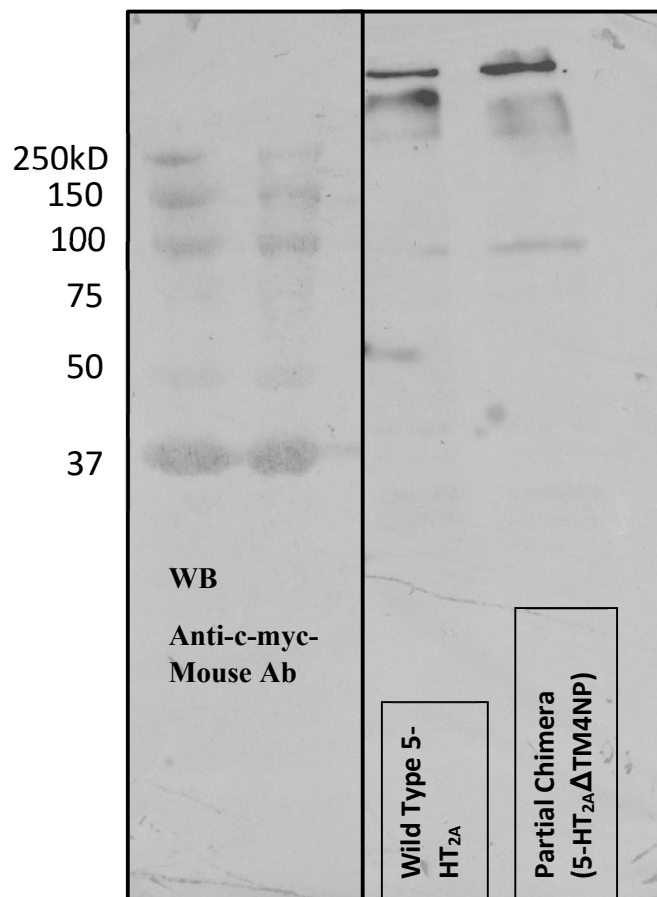


Figure 4. Characterization of Partial TM4 Mutation with Western Blot probing for c-myc-5-HT_{2A}; Ladder merged for Clarity. Lane one samples were previously transfected with WT 5-HT_{2A} receptor. Lane two was loaded with sample previously transfected with the partial mutant (5-HT_{2A}-ΔTM4NP). Primary Antibody: Myc-Mouse Ab. Secondary Antibody: Anti-Mouse Ab.

Finally, figure 4 attempts to characterize the mutant receptor by immunoblot assay. If the mutant is expressed properly, we would expect bands of similar molecular weight for both mutant and wild type receptors. While the immunoblots do not indicate a 53kDa band expected by a monomeric 5-HT_{2A} receptor, one must account for the weight of c-myc (41kDa) and m-cherry tags on 5-HT_{2A} (29kDa) in addition to the possibility of the receptor dimerizing or

oligomerizing. In any case, since the mutant plasmids must be translated by the cell without misfolding or being degraded, the homology of the bands indicated on the western blot suggest that the 5-HT_{2A}-ΔTM4NP chimeric receptor was properly expressed in a manner similar to WT 5-HT_{2A}. The purpose of selecting the partial chimera 5-HT_{2A}-ΔTM4NP in Figure 4 was to ensure that the partial chimera displayed molecular weight bands similar to that of wild type receptors, before creating the full N terminal TM4 chimera 5-HT_{2A}-ΔTM4NF. The full construct and any additional C terminal 5-HT_{2A} chimeras can also be characterized by immunoblot.

There are several additional ways to characterize the mutant receptors in future studies and ensure structural and functional similarity to wild type receptors. To assess whether the mutant receptors bind to ligands in a manner similar to WT 5-HT_{2A}, radioactive Ketanserin binding experiments as outlined in *Moreno, 2016* could be conducted. We would expect similar displacement curves for individually transfected mutant and wild type receptors. The binding between curves of co-transfected wild type receptors and co-transfected mutant 5-HT_{2A} chimeras with mGlu2 should slightly vary depending on the mutation in the chimera. Additionally, experiments considering the cross talk between the protomers of the complex, using LY379268, (an mGlu2 agonist) as described in (Moreno 2016) could be conducted. We would only expect downstream 5-HT_{2A} G_{q/11} calcium signaling in mutant chimeras which are not responsible for breaking the binding interface. Finally, to assess the proper localization of the protein following translation, Immunocytochemistry experiments as those described in *Gonzalez-Maeso, 2008* can be conducted. We would expect a distribution of mutant receptors similar to that of wild type receptors. Mutant receptors could also be co-transfected with mGlu2 before comparing their immunocytochemistry results to those of WT co-transfection.

Conclusions and Future Directions

Findings:

1. Immunoblot experiments conducted in this study are congruent with previous findings that wild type mGlu2 and 5-HT_{2A} coimmunoprecipitate
2. Mutant constructs (5-HT_{2A}-ΔTM4NP and 5-HT_{2A}-ΔTM4NF) created in this study can assess the hypothesis of the paper using the same methods for Co-IP.
 - a. Multiple Co-IP signals can be discriminated by quantification
 - b. Additional chimeric constructs involving the C terminal half of 5-HT_{2A/C} can be made (ex: 5-HT_{2A}-ΔTM4C-P/F) to assess their role in heteromerization.
3. Expression of the partial mutant is verified by similar immunoblot bands displayed for WT 5-HT_{2A} and 5-HT_{2A}-ΔTM4NP western blot.
4. Additional characterization of any constructs produced by this study or those created in the future may include:
 - a. Immunocytochemistry
 - b. [³H] Ketanserin Binding
 - c. Crosstalk experiments (Calcium Assay)

Future Directions if the 5-HT_{2A} N terminal half of TM4 is determined to mediate the heteromeric interface: Photo-Crosslinking Approach

If no co-immunoprecipitation between 5-HT_{2A}-ΔTM4NF and mGlu2 is observed, a photo-crosslinking approach similar to *Grunbeck, 2011 & Valentin-Hansen, 2014*, mentioned earlier, could be used to identify individual amino acids involved in the interface. Recall that while coimmunoprecipitation offers valuable information on the sections of the TM4 involved in

heteromerization, it does not offer evidence for a direct interaction between individual amino acids. Although traditional cysteine cross linking experiments such as ones used to identify a homomeric dopamine receptor (Han, 2009) can be used to assess a direct interaction, the presence of endogenous cysteines mediating the Venus Fly Trap domains of mGlu2 presents a problem; although these cysteines can be mutated to serine or alanine, this substitution seems to alter the structure and dynamics of the mGlu2 homodimer (Koehl, 2019.) While the technical co-transfection of multiple plasmids required by a photo-crosslinking approach may take additional optimization, the approach taken by *Maeso Group, Unpublished* to identify individual amino acids responsible in the mGlu2 receptor, could be modeled to identify the individual residues of 5-HT_{2A} responsible for complex formation without compromising the overall structural integrity of either protomer in the complex.

If the N terminal end residues of TM4 in the 5-HT_{2A} are found to be implicated in the dimeric interface, that is, if coimmunoprecipitation of the mGlu2 and 5-HT_{2A}-ΔTM4NF fails to occur, four separate constructs would need to be created to perform a photo cross linking assay. The four new constructs correspond to the four residues mutated (phenylalanine_{4.43}; leucine_{4.44}; alanine_{4.47}; isoleucine_{4.48}) to create the 5-HT_{2A}-ΔTM4NF construct. The “cocktail” of plasmids transfected, would be refined to introduce a single photoreactive unnatural amino acid corresponding to phenylalanine_{4.43}, leucine_{4.44}, alanine_{4.47}, or isoleucine_{4.48} in each of the four new constructs respectively. These constructs could undergo UV mediated crosslinking, which would reveal whether one or more of the four original amino acids mutated in 5-HT_{2A} form a direct interaction with mGlu2 and participate directly in establishing the heteromeric interface of the 5-HT_{2A}-mGlu2 complex.

The implication of the mGlu2-5-HT_{2A} complex in schizophrenia is clear. The results produced in this study offer valuable constructs to be included in designing future studies. With the mutant constructs created, Co-IP assays can be conducted with the same methods to identify if either disrupts the binding of 5-HT_{2A}-mGlu2. If so, future photo-crosslinking studies can focus on identifying individual amino acids within 5HT_{2A} responsible for the heteromeric interface. If not, additional C terminal TM4 chimeras of the 5-HT_{2A} with aligned residues from 5-HT_{2C} will need to be constructed, coprecipitated, and immunoassayed before verifying the role of the C terminal residues. In any case, gaining a more complete structural understanding of the mGlu2-5-HT_{2A} will be beneficial for therapeutic strategies in Schizophrenia.

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