Role of C121A in mGluR2 homodimeric expression and function

Jong M. Shin
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd

Part of the Cellular and Molecular Physiology Commons, Molecular and Cellular Neuroscience Commons, and the Pharmacology Commons

© Jong Myoung Shin

Downloaded from
https://scholarscompass.vcu.edu/etd/5576

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
Role of C121A in mGluR2 homodimeric expression and function

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science at Virginia Commonwealth University.

by

Jong Myoung Shin
Bachelor of Science, Pennsylvania State University

Director: Javier Gonzalez-Maeso, PhD
Associate Professor, Physiology and Biophysics Department

Virginia Commonwealth University
Richmond, Virginia
August, 2018
Acknowledgment

It was my greatest honor to have worked with Dr. Javier Gonzalez-Maeso and the brilliant minds in his lab to produce this work. The process of reaching this point encompassed countless trials-and-errors which left me speechless from time to time. It was frustrating and yet exuberating to advance my way up to the scientific truth. Here, I reaffirmed myself of my future aspirations and gained the confidence that I am able to achieve my goals in life.

I cannot thank enough Dr. Gonzalez-Maeso for not only taking me in as his pupil but also for treating me like his family. His utmost quality as a scientist has drawn a flock of talented individuals, which concurrently set high standards in his work environment. Owing to that, I have never felt so small in my life. And yet, my admiration for him unconsciously made me mimic the way he talks, thinks, acts, and communicates, which ultimately made me a better scholar. At the end of this training, I realized that I fully integrated into these brilliant group of scientists that I respect with my whole heart. I am proud to have received teachings from my greatest mentor, and his legacy will always carry on with me wherever I go. He has indeed changed my life, and I am most certainly grateful for those changes.

Dr. Sammanda Ramamoorthy and Dr. Gea-Ny Tseng have generously spent their valuable time to guide me through my research endeavor. Despite their extreme busy schedule, they have always made availability for the sake of my training. With their knowledge and experience, I was able to find my answer when I encountered stalemate during my research. I am personally indebted to their supports, and I sincerely thank you for serving on my thesis committee.
It is imperative to mention here the names of my colleagues who contributed to this work as much as I did, if not more. This is not just to acknowledge my colleagues, but it is also to serve as an important reminder for me in the future that I did not get there alone.

Dr. Mario de la Fuente Revenga has a warm heart and is my great compassionate friend. He painstakingly trained me on his automated head twitch response behavior apparatus he invented. The last part of the thesis on the HTR experiment would not have existed without his ingenuity, and that section is dedicated to his generosity. Dr. Urjita Shah is an exceptional scientist by all means. Despite her young age, I learned tremendously from her. She has been taking care of me and my research like how a mother bird would care for her eggs. She has been involved in most of my thesis as she is also studying the crosstalk between 5-HT$_2$A and mGluR2. Dr. Salvador Sierra is relatively a new member in our lab holding MD and PhD degrees. He advised me on microbiology and molecular biology aspect of the thesis. He also specializes in proximity ligation assay which he sees the applicable value in the work of mGluR2 homodimerization.

Mr. Rudy Toneatti is one of the most selfless person I have ever encountered in my life. He would stay past midnight only to help me with my research after working since 8 a.m. He is probably the person who is most technically involved in my work from site-directed mutagenesis to generating stable cell line. I am extremely thankful for his assistance and mentorship in the lab. Mr. Justin Saunders is a MD/PhD candidate at VCU who is extraordinarily intelligent and shows unconditional kindness to other people. He always amazes me with his novel ideas that no ordinary scientist can engender. He has superb working memory and very quick thinking skills. I am personally curious where he will end up in this field. He immensely helped me with the
western blot. Mr. Doan On is another brilliant and extremely literate scientist with over 10 years of experience in research. Owing to his expertise and kindness, he was initially the first responder to my request for help. His knowledge base is vastly broad, and I benefitted from that in the work of biochemical assays, binding assays, and cell culture techniques. He is currently half way through his PhD program.

Mr. Travis Cuddy and I started this Master’s program in Physiology and Biophysics together. He quickly gained his expertise in GTPγS binding assay and taught me how to run the assay most efficiently and properly. He is very talented and plans to go to medical school. I would also like to thank Ms. Cassandra Dunn for her technician work. She has been a great friend, and I will always miss her as long as I can remember.

I would like to ink the names of my parents, Un Shin and Young Ran Kim, on my thesis where my effort for success is carved within. I could not ask for a better couple to give life to my soul and nurture me throughout my existence. As my creators and eternal supporters, I will love them with all my heart and the rest of my life will be devoted to them.

Lastly, and probably the most importantly, I would like to thank my little sister, Jaime Shin, who has given me critical reviews and proofread my writings including this thesis. Her expertise in English helped me throughout my career, and it would not have been possible to be where I am without her help. I am grateful that she has spent significant amount of time to edit this thesis despite her busy schedule as a law student.

Once again, I would like to thank everyone who were directly and indirectly involved in creating this thesis. I hope that this work would positively impact at least a small part of my beloved scientific community.
Table of Contents

ACKNOWLEDGMENT 2

TABLE OF CONTENTS 5

LIST OF FIGURES 7

LIST OF ABBREVIATIONS 8

ABSTRACT 10

CHAPTER 1. INTRODUCTION 11

1.1. G PROTEIN-COUPLED RECEPTORS 11
1.1.1. HETEROTRIMERIC G PROTEINS 11
1.1.2. CLASS C GPCR 12
1.2. METABOTROPIC GLUTAMATE RECEPTOR II (mGLuR2) 15
1.2.1. GLUTAMATE HYPOTHESIS OF SCHIZOPHRENIA 15
1.2.2. HOMO-DIMERIZATION OF mGLuR2 16
1.2.3. CONSERVED CYSTEINE RESIDUE AT POSITION 121 16
1.3. 5-HT2A-mGLuR2 HETEROMERIZATION 20
1.3.1. POTENTIAL mGLuR2 HOMODIMER INTERFERENCE IN THE HETEROMERIZATION 20
1.3.2. HETEROMER CROSSTALK IN A MOUSE MODEL 21

CHAPTER 2. OBJECTIVES 23

CHAPTER 3. MATERIALS AND METHODS 25

3.1. CONSTRUCTION OF mGLuR2-C121A PLASMID 25
3.2. HEK-293 TRANSIENT TRANSFECTION OF mGLuR2-C121A PLASMID 25
3.3. GENERATION OF STABLY EXPRESSING CELL LINE 26
3.4. IMMUNOFLUORESCENCE MICROSCOPY 27
3.5. MEMBRANE PREPARATION 28
3.6. WESTERN BLOT 28
3.7. [3H]LY341495 SATURATION BINDING ASSAY 29
3.8. [35S]GTPyS BINDING ASSAY 29
3.9. AUTOMATED HEAD TWITCH BEHAVIOR TEST 30
3.10. DATA ANALYSIS 31
CHAPTER 4. RESULTS

4.1. VISUALIZATION OF RECEPTOR EXPRESSION AND ITS SUBCELLULAR LOCALIZATION 33
4.2. mGLuR2 MONOMER-DIMER EQUILIBRIUM ASSESSMENT BY WESTERN BLOT 38
4.3. CHANGE IN BINDING POTENTIAL IN [3H]LY341495 SATURATION BINDING ASSAY 41
4.4. [35S]GTPyS FUNCTIONAL ASSAY INDICATES AFFECTED G-PROTEIN COUPLING 43
4.5. mGLuR2 MODULATION IN 5-HT2A-DEPENDENT PSYCHOSIS MOUSE MODEL 46

CHAPTER 5. GENERAL DISCUSSION 53

REFERENCES 60

VITA 63
# List of Figures

<table>
<thead>
<tr>
<th>Figure Number</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematics of GPCR signal cascade and downstream effects.</td>
<td>13</td>
</tr>
<tr>
<td>1.2</td>
<td>Diagram of Class C GPCR as a homodimer.</td>
<td>14</td>
</tr>
<tr>
<td>1.3</td>
<td>$G_{i/o}$ dependent adenylyl cyclase inhibition mechanism.</td>
<td>18</td>
</tr>
<tr>
<td>1.4</td>
<td>mGluR2 homodimer.</td>
<td>19</td>
</tr>
<tr>
<td>1.5</td>
<td>Schematics for different actions of heteromer mechanism.</td>
<td>22</td>
</tr>
<tr>
<td>3.1</td>
<td>Set up for NI DAQ data acquisition system.</td>
<td>32</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Immunofluorescence microscope images without permeabilization.</td>
<td>34</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Immunofluorescence microscope images with permeabilization.</td>
<td>35</td>
</tr>
<tr>
<td>4.1.3</td>
<td>Transfection optimization.</td>
<td>36</td>
</tr>
<tr>
<td>4.2</td>
<td>Spinning disc confocal microscopy.</td>
<td>37</td>
</tr>
<tr>
<td>4.3</td>
<td>Western blot of the membrane preparation.</td>
<td>39</td>
</tr>
<tr>
<td>4.4</td>
<td>Quantified western blot band intensity by densitometer.</td>
<td>40</td>
</tr>
<tr>
<td>4.5</td>
<td>Slightly lower binding potential of the mGluR2-C121A.</td>
<td>42</td>
</tr>
<tr>
<td>4.6.1</td>
<td>Decreased $G_i$ coupling of the mGluR2-C121A.</td>
<td>44</td>
</tr>
<tr>
<td>4.6.2</td>
<td>Quantitative analysis of the</td>
<td>45</td>
</tr>
<tr>
<td>4.7.1</td>
<td>Positive 5-HT$_{2A}$ modulation by mGluR2 deactivation.</td>
<td>47</td>
</tr>
<tr>
<td>4.7.2</td>
<td>Positive 5-HT$_{2A}$ modulation by mGluR2 deactivation.</td>
<td>48</td>
</tr>
<tr>
<td>4.8.1</td>
<td>Negative 5-HT2A modulation by mGluR2 activation.</td>
<td>49</td>
</tr>
<tr>
<td>4.8.2</td>
<td>Negative 5-HT2A modulation by mGluR2 activation.</td>
<td>50</td>
</tr>
<tr>
<td>4.9.1</td>
<td>LY404039 incubation for 30 minutes</td>
<td>51</td>
</tr>
<tr>
<td>4.9.2</td>
<td>LY404039 incubation for 30 minutes</td>
<td>52</td>
</tr>
</tbody>
</table>
List of Abbreviations

5-HT$_{2A}$  5-hydroxy-tryptamine subtype 2A receptor  
Bps  Base pairs  
BSA  Bovine serum albumin  
C$^{121}$  Cysteine at position 121  
cAMP  cyclic adenosine monophosphate  
CASR  Single calcium sensing receptor  
CNS  Central nervous system  
DMEM  Dulbecco’s modified Eagle’s medium  
DOI  2,5-dimethoxy-4-iodoamphetamine  
dPBS  Dulbecco's phosphate-buffered saline  
ER  Endoplasmic reticulum  
G-proteins  Guanine nucleotide-binding proteins  
GABA  Gamma-aminobutyric acid  
GTP  Guanosine triphosphate  
HA-tag  Human influenza hemagglutinin  
HCl  Hydrochloric acid  
HEK-293  Human embryonic kidney 293 cell line  
HTR  Head twitch response  
IP$_3$  Inositol triphosphate  
kDa  Kilo-Dalton  
LSD  Lysergic acid diethylamide  
mGluR  Metabotropic glutamate receptor  
MW  Molecular weight  
P/S  Penicillin and streptomycin  
PBS  Phosphate buffer solution
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
Abstract

ROLE OF C121A IN MGLUR2 HOMODIMERIC EXPRESSION AND FUNCTION

By Jong Myoung Shin, Bachelors of Science

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2018

Director: Javier Gonzalez-Maeso, PhD
Associate Professor, Physiology and Biophysics Department

The group II metabotropic glutamate receptors are known for their involvement in various psychiatric disorders. The mGluR2 in particular is linked with etiology of schizophrenia especially in the context of crosstalk with 5-HT$_{2A}$. Thus, the mGluR2 has attracted attentions for its potential therapeutic applications. Despite numerous physiological evidences on the actions of mGluR2, its mechanism is still unclear to this day. It is partially due to the lack of understanding in characteristics of mGluR2 homodimer which is its functionally active form. Therefore, the characterization of dimeric interaction serves as a foundation to advanced understanding of the role of mGluR2. On that note, the role of the conserved cysteine residue (C$_{121}$) in the ligand binding domain of mGluR2 has been evaluated in this study as they are known to play a critical part in homodimer formation. Collectively, C$_{121}$ has been shown to affect the dimerization, subcellular localization, and pharmacokinetics of mGluR2. Lastly, the effect of mGluR2 on mouse behavior was examined in a partial effort to elucidate its role in crosstalk with 5-HT$_{2A}$. 
Chapter 1. Introduction

1.1. G Protein-Coupled Receptors

G Protein-Coupled Receptors (GPCRs) comprise a large family of membrane receptors modulated by extracellular stimulation transducing intracellular signal cascade to regulate physiological processes in eukaryotic organism (Prazeres, 2015). There are myriad receptor ligands including chemical compounds, hormones, neurotransmitters, small molecules, and peptides involved in vital biological processes such as cell proliferation, migration, and inflammation (Tadagaki, 2012). These receptors are ubiquitously expressed throughout the system. Owing to their abundance, they are responsible for a numerous physiological abnormalities including various psychiatric disorders (Komatsu, 2015). For such reason, the researches to better understand functionalities of GPCRs continuously gained tractions in the past several decades.

1.1.1. Heterotrimeric G Proteins

GPCRs are associated with guanine nucleotide-binding proteins (G-proteins) that are either monomeric or heterotrimeric (Flock, 2015). Of those two major classes, the heterotrimeric G-proteins are particularly important in cell signaling and receptor trafficking, hence it is an attractive research subject from a pharmacological perspective. As the name suggests, heterotrimeric G-proteins involve three distinctive proteins that are membrane bound: alpha (Gα),
beta ($G_\beta$), and gamma ($G_\gamma$). These G-proteins act as molecular switches for pertinent GPCRs turning the receptors on or off by binding either GTP or GDP, respectively. Depending on which GPCRs they are associated with, the $G_\alpha$ proteins can be subdivided into four main subclasses ($G_\alpha_s$, $G_\alpha_i$, $G_\alpha_q$, and $G_\alpha_{12/13}$) based on the protein sequence and the effectors they activate (Beaulieu & Gainetdinov, 2011). Upon GTP hydrolysis, these G-proteins detach from the GPCR into two separate units as $G_\alpha$ and $G_\beta\gamma$, which individually activate their corresponding effectors such as adenylyl cyclases, phospholipases, and ion channels (Figure 1.1).

1.1.2. Class C GPCR

GRAFS classification groups GPCRs into five main families in human: Glutamate (G/Class C), Rhodopsin (R/Class A), Adhesion (A/Class B), Frizzled (F/Class F), and Secretin (S/Class B) (Schioth, 2005). Within these families, the class C GPCRs are known for their large extracellular domain serving as orthosteric binding sites known as the venus flytrap domain (Figure 1.2). Additionally, they are known to constitutively form strict dimers as a part of their activation mechanism (Levitz, 2016; Xue, 2015). The class C GPCRs consist of eight metabotropic glutamate receptors (mGluRs), two $\gamma$ aminobutyric acid (GABA) receptors, single calcium sensing receptor (CASR), and sweet and amino acid taste receptors, pheromone receptors, and odorant receptors (Pin, 2003). As the L-glutamate is the most prevalent neurotransmitter in the excitatory synapses in the mammalian CNS, the mGluRs are of particular interest in its therapeutic utility to treat various neurological and psychiatric disorders.
Figure 1.1 Schematics of GPCR signal cascade and downstream effects. Examples of different sensory inputs are listed in the box. Four discrete G-proteins are depicted in the diagram above. The following blue arrows indicate the corresponding targets being activated by each G-protein. The bottom-most box contains a partial list of the physiological effects occurred by these G-proteins (Schou, 2015).
Figure 1.2 Diagram of Class C GPCR as a homodimer. Venus flytrap domains are located at the extracellular ligand binding domains serving as orthosteric binding sites. The proposed mechanism distinguishes three different state of the homodimer based on ligand availability in the binding sites (Niswender, 2010).
1.2. Metabotropic glutamate receptor II (mGluR2)

The mGluRs can be further categorized into three distinct groups (group I, II, III) according to G-protein coupling, ligand selectivity, and sequence homology (Niswender, 2010). The mGluR2 belongs to the group II of these sub-classes along with mGluR3. The mGluR2 are characteristically coupled to $G_{i/o}$ alpha proteins inhibiting activities of adenylyl cyclase, which in turn decrease intracellular cAMPs (Figure 1.3). This regulation of the secondary messenger modulates neurotransmitter synthesis, storage, release, receptor sensitivity, neuronal growth and differentiation (Duman & Nesler, 1999).

1.2.1. Glutamate hypothesis of schizophrenia

Glutamatergic neurotransmission is required in most of the normal brain functions in that it is also responsible for many neuropathological disorders such as schizophrenia. There are numerous reports suggesting that disruptions of neurotransmitter communication contribute to such clinical conditions (Brichta, 2013; Marecos, 2014). The group II mGluRs are of particular interest in schizophrenia research due to the glutamate hypothesis, which speculates dysfunction of glutamatergic neurotransmission to be involved in the etiology of schizophrenia (Javitt, 2012). With the basis of the hypothesis, mGluR2 and mGluR3 have been investigated for their possible influences from different parts of post-mortem human brain. One particular study compared the expression levels between affected and unaffected schizophrenia brain samples. The expression levels of the group II mGluRs showed lower level of mGluR2 in the prefrontal cortex and
cerebellum while there was not a significant change in the mGluR3 levels (Gonzalez-Maeso, 2008).

1.2.2. Homo-dimerization of mGluR2

Along with the other groups of mGluRs, mGluR2 takes a functional form as a strict homodimer. Hence, there may be a mixed population of monomeric and dimeric mGluR2 in the cells that expresses this protein. Although the comprehensive mechanism for this dimerization is still unclear, a number of studies previously suggested the involvement of different transmembrane interactions as a part of possible mechanism (Moreno, 2016; Xue, 2014). Furthermore, another report showed a cooperativity as a homodimer mechanism via communication between two ligand binding domains (LBDs). G-protein activation requires dimerization of full length mGluRs and is partially mediated by an intersubunit disulfide bridge between the LBDs (Levitz, 2016). However, the latter claim is still subject to questions as there has not been a thorough investigation on this particular matter, which is a compelling idea for discussion as it is the only known residue that participates in an extracellular intersubunit covalent bonding. Thus, this topic alone deserves experimentation of its own.

1.2.3. Conserved cysteine residue at position 121

The intersubunit disulfide bridge between the LBDs is formed by two cysteine residues at position 121. It is the only known cysteine residue that covalently connects two mGluR2s from the extracellular domains although there are several other cysteines that are available for
disulfide linkage (Niswender, 2010; Rondard, 2006). This residue is conserved throughout all mGluRs and is known to play its role in stabilizing the mGluR2 homodimer structure in conjunction with hydrophobic interaction between the LBDs (Levitz, 2016, Figure 1.4). However, the precise functions of C^{121} is still controversial due to the lack of thorough investigation on this topic. This calls upon an inquiry for further study on this specific cysteine residue to elucidate its effect on dimerization, functionality, and trafficking of mGluR2.
Figure 1.3 $G_{i/o}$ dependent adenylyl cyclase inhibition mechanism. Activated $G_{i/o}$ protein inhibits adenylyl cyclase preventing the conversion of ATP into cAMP. Decreased cAMP level ultimately prevents release of glutamate from the presynaptic terminal of neurons (Li, 2015).
Figure 1.4 mGluR2 homodimer. Two mGluR2 protomers come together to form a quaternary structure with multiple contacts at ligand binding domains and transmembrane domains. The C\textsuperscript{121} contributes to the stability of the homodimer structure via extracellular disulfide bridge (Moller, 2017).
1.3. 5-HT$_{2A}$-mGluR2 Heteromerization

Heteromerization between different receptors is a unique physiological phenomena that it can promote or attenuate original signal transduction which can lead to a new signaling pathway in some cases. (Albizu, 2010; Figure 1.5). Namely, a number of evidences corroborates such existence of class A GPCR heterodimers, although the topic is still in debate (Franco, 2016; Moutkine, 2017). The serotonin 5-HT$_{2A}$, a class A GPCR, and the mGluR2 are known to heterodimerize, and they have been linked to the pathophysiology of schizophrenia (Gonzalez-Maeso, 2008; Levitz, 2016; Moreno, 2016; Xue 2015). In the HEK-293 cells that co-express 5-HT$_{2A}$ and mGluR2, a synthetic mGluR2 agonist could increase the intracellular calcium level with the absence of 5-HT$_{2A}$ agonist suggesting that the crosstalk was initiated from the activation of mGluR2 inducing downstream signal transduction via 5-HT$_{2A}$-coupled G$_{q}$ alpha protein, which activates the phospholipase C that triggers inositol triphosphate (IP$_{3}$) calcium signaling pathway (Moreno, 2016).

1.3.1. Potential mGluR2 homodimer interference in the heteromerization

As two mGluR2 protomers are covalently stabilized by $C^{121}$, it is possible that the presence of homodimer can potentially interfere with the formation of 5-HT$_{2A}$-mGluR2 heteromer. While these receptors can theoretically form a multi-complex oligomer between two homodimers, a few reports suggest a possible combination of heteromer potentially consisting of 5-HT$_{2A}$ and mGluR2 monomers (Baki, 2016; Moreno, 2018). However, it is difficult to propose a mode of interaction without an explicit structural information of the homodimer or heteromer,
or a properly devised mechanism. In order to elucidate this crosstalk mechanism, it calls for the need for an in-depth investigation on the role of C121 as it may constitute a crucial part of forming a homodimer.

1.3.2. Heteromer crosstalk in a mouse model

Hallucination is one of the hallmark positive symptoms of schizophrenia (Lysaker, 1999). In addition, increased expression of 5-HT2A and decreased expression of mGluR2 in the prefrontal cortex of the human post-mortem sample were reported (Gonzalez-Maeso, 2008). Thus, many serotonergic hallucinogens such as lysergic acid diethylamide (LSD) and 2,5-dimethoxy-4-iodoamphetamine (DOI) are used to emulate psychotic behaviors in mice and rats, which exhibits peculiar side-to-side head movements, known as head twitch response (HTR), upon receiving the drugs (Willins, 1997). As the LSD-induced hallucination resembled positive symptoms of schizophrenia, 5-HT2A activation in the prefrontal cortex led to the development of psychosis model in rodents. Therefore, 5-HT2A signaling efficiency was coupled to a prediction of vulnerability to psychiatric illness (Gonzalez-Maeso & Meana, 2006). Furthermore, the activation of mGluR2 seems to modulate the effect of 5-HT2A-dependent hallucinogens. In that regard, 5-HT2A-mGluR2 heteromer was explored as a therapeutic target for schizophrenia in a mouse model by attempting to inhibit the HTR from mGluR2 activation (Benvenga, 2018).
Figure 1.5 Schematics for different actions of heteromer mechanism. (a) Depiction of receptor desensitization upon binding corresponding ligand. (b) Heteromerization can affect the ability of ligand binding. Two receptors can show positive (+) or negative (-) cooperativity, which can either increase or decrease affinity for ligands. (c) Heteromer can engender a new signal pathway and produce different physiological responses that were not possible in monomeric or homodimeric forms (Albizu, 2010).
Chapter 2. Objectives

The mGluR2 is considered an important piece to a variety of neuropathological disorders in the range from depression to Alzheimer’s disease (Duman, 2018; Lee, 2009). This receptor has been in the center of conversation as a key element for the glutamate hypothesis of schizophrenia. Since the evidence for 5-HT$_{2A}$-mGluR2 heteromer existence was published, this heteromer revealed a new scope of understanding the mechanism of this psychotic disorder and attracted attention as a potential therapeutic target. While the crosstalk between these receptors were biochemically demonstrated previously (Gonzalez-Maeso, 2008), the mechanism behind the process has not been clearly elucidated. It is partially due to the class C GPCRs’ ability to constitutively form a strict dimer. Hence, the study of mGluR2 homodimer is a crucial part to illustrate comprehensive picture of the crosstalk mechanism. In order to better understand the process of mGluR2 homodimer formation, C$^{121}$ holds a great value as this conserved cysteine residue readily forms extracellular disulfide bridge between two LBDs, which stabilizes overall homodimer structure. To further investigate the role of C$^{121}$ in homodimer formation and the impact of mGluR2 modulation of 5-HT$_{2A}$ in a schizophrenia mouse model, the following four aims were employed:

1. Evaluate the effect of C121A on subcellular localization of mGluR2 in HEK-293 cells.
2. Investigate the changes in the binding affinity and binding potential of the C121A mutant.
3. Assess the mGluR2 monomer-dimer equilibrium between the WT and C121A mutant.

4. Examine the G\textsubscript{i} coupling to assess the functionality of mGluR2

5. Characterize the modulating effect of mGluR2 on 5-HT\textsubscript{2A}-dependent head twitch behavior in animal models of psychosis.
Chapter 3. Materials and Methods

3.1. Construction of mGluR2-C121A plasmid

Previously constructed pcDNA3.1(+)-HA-mGluR2-mCitrine plasmid by Dr. Javier Gonzalez-Maeso was used as a template to introduce a single point mutation via QuikChange II Site-Directed Mutagenesis Kit according to the protocol of manufacturer (Agilent). All PCR assays were performed with PfuUltra High-Fidelity DNA polymerase (Agilent). Cycling conditions were 16 cycles of 95°C for 30s, 55°C for 1 min, and 68°C for 9 minutes with an initial denaturation step of 95°C for 30s. Forward (5’-GGCTCACGCCACATCGCGCCCGACGGCTCTTAT-3’) and reverse (5’-ATAAGAGCCGTCGGGCGCGATGTGGCGTGAGCC-3’) mutagenesis primers were ordered (Agilent). The PCR product was transformed into XL1-Blue Competent Cells according to the protocol of manufacturer (Stratagene). The selected bacterial colonies were cultured in the LB-ampicillin medium for 18 hours, and the plasmids were purified using the QIAGEN plasmid Miniprep and Maxiprep kits. Purified plasmids were digested with NheI and XbaI restriction enzymes for band analysis. Band weight confirmed plasmid samples were sequence verified as a final step of quality control (Eurofin).

3.2. HEK-293 transient transfection of mGluR2-C121A plasmid

HEK-293 cells were maintained in the Dulbecco’s modified Eagle’s medium (DMEM; contains 4.5 g/L glucose) supplemented with 10% (v/v) dialyzed fetal bovine serum (dFBS) and
1% (v/v) P/S at 37°C in a 5% CO2 humidified atmosphere. dFBS was used to prevent contamination by glutamate from using undialyzed FBS. Transfection for immunofluorescence microscopy and western blot was performed with PEI using 3µg of plasmid materials incubated for 24 hours. For the rest of application, transfection was performed with PEI using 10µg of plasmid materials without P/S for 24 hours. Lower amounts of DNA materials were used for the former assays due to their sensitivity to over-expression of the receptors.

3.3. Generation of stably expressing cell line

HEK-293 cells were transiently transfected as described in 3.2. After 24 hours incubation, the cells were split into 1:200, 1:400, and 1:600 dilutions and allowed for them to stabilize in DMEM (contains 4.5 g/L glucose) supplemented with 10% (v/v) dFBS and 1% (v/v) P/S at 37°C in a 5% CO2 humidified atmosphere. After 24 hours, the medium was replaced with the DMEM supplemented with hygromycin B (250 µg/ml) for selection. Until adequate number of foci was grown, the culture was continuously supplied with a new DMEM containing hygromycin B. Once foci matured, they were screened for fluorescence under the microscope. The medium was removed and washed with pre-warmed Dulbecco's phosphate-buffered saline (dPBS). Foci were circumscribed with cloning rings and completely isolated from their surroundings. The cloning rings were adhered to the plate with 2% agarose gel. Foci were trypsinized (Thermofisher) and transferred to 96-well plates for morphology screening.
3.4. Immunofluorescence microscopy

HEK-293 cells were grown on poly-D-lysine-treated glass coverslip (number 0 thickness) and transiently transfected to express either HA-mGluR2-mCitrine or HA-mGluR2-C121A-mCitrine. After 24 hours transfection period, the medium was removed and the cells were fixed with 2% PFA for 15 minutes followed by three 10 minutes washes with PBS. For permeabilization, 0.2% Triton-X-100 was treated for 10 minutes at RT followed by three 10 minutes washes with PBS. To reduce the unspecific binding of antibodies, the cells were blocked with BSA prior to the incubation with antibodies. The cells were incubated with the mouse anti-HA tag antibody (Cell Signaling) for 60 minutes in RT followed by three 5 minutes washes with PBS. For additional block after primary antibody incubation, the cells were washed with BSA three times for 5 minutes. The cells were incubated in dark with the rabbit anti-mouse antibody labeled with Alexa Fluor 594 (ThermoFisher) for 60 minutes in RT followed by three 5 minutes washes with PBS. For nuclei staining, the cells were incubated with Hoechst 33342 dye solution (ThermoFisher) in dark at RT for 5 minutes followed by three 5 minutes washes with PBS. Residual PBS is gently washed with distilled water. The coverslip was inverted onto Vectashield antifade on a microscope slide and sealed with nail polish. Zeiss LSM 710 confocal fluorescent microscope was used to resolve the prepared slides.
3.5. Membrane preparation

The cells were harvested by centrifugation (1,000 g, 5 minutes, 4°C) in cold dPBS. dPBS was aspirated and the cell pallet was frozen at least for an hour. The cells were thawed and homogenized in an ice-cold Tris buffer (50 mM Tris-HCl, pH 7.4). The homogenate was centrifuged (1,000 g, 5 minutes, 4°C) to remove nuclei, and the supernatant was collected and centrifuged (40,000 g, 10 minutes, 4°C). The pellet was washed with 5 ml of ice-cold Tris buffer and recentrifuged (40,000 g, 5 minutes, 4°C). The resultant pellet was stored at -80°C for future use.

3.6. Western blot

The amount of protein in the membrane preparation was estimated using Bradford assay. 20 ug of proteins were chemically reduced with 2-mercaptoethanol. The protein sample was resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes by electrophoresis overnight. The membranes were blocked with blocking buffer containing milk and BSA for 1 hour at RT, which was then immunoblotted with primary antibody (mouse anti-HA, 1:1000, Abcam) overnight at 4°C. Subsequently, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (rabbit anti-mouse, 1:5000, Abcam) for 1 hour at RT. Immunoreactivity was detected from the protein sample by the enhanced chemiluminescence system (SuperSignal WestPico #34080, Thermofisher) according to the protocol of manufacturer. The resultant western blot films were analyzed for band intensity by densitometry using the GelQuantNET software (v. 1.8.2.).
3.7. \(^3\)HLY341495 saturation binding assay

Various concentrations of \(^3\)HLY341495 (American Radiolabeled Chemicals (ARC). Cat# ART-1439) from 0 to 15 nM were incubated with 5 ug of membrane proteins per each well on ice for 60 minutes in a total volume of 200 uL. Subsequently, the concentrations of \(^3\)HLY341495 used were corrected according to radioactivity measured by TRI-CARB 4910TR 110 V Liquid Scintillation Counter (PerkinElmer). The membrane proteins were re-suspended in the phosphate buffer (10 mM K\(_2\)HPO\(_4\), 1 mM KH\(_2\)PO\(_4\), 100 mM KBr, pH 7.6). Non-specific binding was determined for each concentration of radioligand in the presence of 1 mM L-glutamate. For harvesting radioligands, Unifilter-96 cell harvester was used (PerkinElmer). The 96-well plates of the membrane incubation were harvested on the GF/A filter papers soaked in 0.5% PEI. The filter paper was dried at 65°C for 30 minutes. Dried filter paper was soaked with 40 ml Microscint-20 cocktail, which was then counted on Perkin Elmer Microbeta2 2450 microplate counter.

3.8. \(^35\)S\(\text{GTP}_\gamma\)S binding assay

The membrane proteins were prepared as indicated in Section 3.5. The prepared membranes were re-suspended in the assay buffer (20 mM HEPES, 100mM NaCl, 3mM MgCl\(_2\), 1 mM EGTA, pH 7.6) accordingly to accommodate 5 ug per each well. 0.5 nM \(^35\)S\(\text{GTP}_\gamma\)S (PerkinElmer. Cat# NEG030H250UC) and 50 uM GDP were used along with various concentrations of LY379268 ranging from 0 to 10 uM to incubate membrane proteins in 30°C for
2 hours in a total volume of 200 uL per each condition. Non-specific binding was determined using 10 uM non-radioactive GTPγS. For harvesting radioligands, Unifilter-96 cell harvester was used (PerkinElmer). The 96-well plates of the membrane incubation were harvested on the GF/A filter papers soaked in the assay buffer. The filter paper was dried at 65°C for 30 minutes. Dried filter paper was soaked with 40 ml Microscint-20 cocktail, which was then counted on Perkin Elmer Microbeta2 2450 microplate counter.

3.9. Automated head twitch behavior test

Small magnets were previously implanted on the skull of adult C57BL/6 mice by Dr. Mario de la Fuente Revenga. The mice were weighed and calculated for proper drug dosing. These mice were placed in copper-wire-wrapped plastic chambers to capture their HTRs from the NI DAQ data acquisition system (National Instruments; Figure 3.1). First, the baseline HTR of these mice were captured for 15 minutes as a habituation process. Appropriate test drugs were intraperitoneally administered followed by HTR measurement for a designed time period. After the drug interval, the head twitch was induced by DOI and captured for a designed time period. The mice underwent washout period of at least 1 week prior to any experiments. The experiments were repeated by crossing the group.
3.10. Data Analysis

In Bradford assay, the total protein contents of the samples were estimated by interpolating the integrated optical density of different concentrations of BSA proteins. Two binding assays conducted in this thesis, \[^{3}{\text{H}}\]LY341495 saturation assay and \[^{35}{\text{S}}\]GTP\(\gamma\)S functional assay, were analyzed with GraphPad Prism software using a one-site nonlinear regression model to plot the graph. The vertical axis of \[^{3}{\text{H}}\]LY341495 saturation binding assay was reported as femtomole of radioligands bound to mg of total proteins. This allowed the calculation of equilibrium dissociation constants (K\(_d\)) and the maximal number of binding sites (B\(_{\text{max}}\)), which were used to calculate the binding potential as described in Chapter 4.3. The vertical axis of \[^{35}{\text{S}}\]GTP\(\gamma\)S functional binding assay was reported as relative fold increases from the basal activity. Corresponding statistical comparison shown on Figure 4.6.2 was conducted using unpaired t-test between mGluR2-WT and mGluR2-C121A.

For assessing the automated head twitch behavior data, the GraphPad Prism software was used to plot the data in bar graphs. Multiple t-tests were used to statistically compare the difference in two experimental groups (vehicle versus LY341 or LY404 groups). Statistical significance of experiments involving multiple groups with two experimental conditions was assessed by two-way ANOVA followed by Sidak’s post hoc test. All data are presented as mean \(\pm\) standard error of the mean (SEM). All statistical comparisons were made at a p-value of 0.05 unless stated otherwise.
Figure 3.1 Set up for NI DAQ data acquisition system. Two acquisition systems are simultaneously being operated. Copper wires (red) are wrapped around the plastic chamber where the mice were enclosed inside during the behavioral test. The chamber was closed with mouse cage covers to prevent escape.
Chapter 4. Results

4.1. Visualization of receptor expression and its subcellular localization

The expression and subcellular localization of the HA-tagged mGluR2-WT and mGluR2-C121A were evaluated under the confocal fluorescence microscope. The HEK-293 cells were transiently transfected with either mGluR2-WT or mGluR2-C121A, thus the reason for untransfected population of cells in the image. In the case of non-permeabilized cells, the antibody seems to bind on the surface of the cells that expresses either receptors (Figure 4.1.1). When the cells were treated with Triton-X-100, a chemical detergent, to permeabilize the plasma membrane to allow antibodies to bind receptors that are subcellularly located inside the cell, the co-localization between mCitrine (green) and anti-GFP (red) was more distinctively visible (Figure 4.1.2). Also, there was a noticeable number of burst cells that had lost fluorescence from mCitrine while retaining signals from the antibody appearing in red (not shown). Comparing the cells with intact structural integrity, the mGluR2-C121A seems to show more accumulation inside the cells compare to the wild type receptor. In regards to the plasma membrane, a qualitatively comparable fluorescence around the cells is suggestive of the receptor presence at the cell surface even with the permeabilization.

Next, the expression profile of the stable cell lines that expresses either mGluR2-WT or mGluR2-C121A were assessed, thus the spinning disc confocal microscopy was employed for higher resolution imaging (Figure 4.2). Similar to the results from the immunofluorescence microscopy, both receptors seem to traffic to the cell surface. In contrast, the subcellular aggregates of the C121A mutant was not detectable in this live cell imaging.
Figure 4.1.1 Immunofluorescence microscope images without permeabilization. HEK-293 cells were transiently transfected with either mGluR2-WT or mGluR2-C121A. The nuclei were stained with Hoechst dye (blue). The proteins were visualized by the conjugated mCitrine fluorescence protein (green) and the anti-HA antibody labeled with fluorophore (red). The wild type mGluR2 (top row) is compared with mGluR2 with C121A mutation (bottom row).
Figure 4.1.2 Immunofluorescence microscope images with permeabilization. HEK-293 cells were transiently transfected with either mGluR2-WT or mGluR2-C121A. The nuclei were stained with Hoechst dye (blue). The proteins were visualized by the conjugated mCitrine fluorescence protein (green) and the anti-HA antibody labeled with fluorophore (red). The wild type mGluR2 (top row) is compared with mGluR2 with C121A mutation (bottom row).
Figure 4.1.3 Transfection optimization. (A) 3ug plasmids with PEI (B) 10ug plasmids with PEI (C) 10ug plasmids with PEI (without P/S) (D) 10ug plasmids with PEI (without P/S) (E) 6ug plasmid with Lipofectamine 2000 (without P/S) (F) 12ug plasmids with Lipofectamine 2000 (without P/S)
Figure 4.2 Spinning disc confocal microscopy. Live cell images of the HEK-293 cells stably expressing either mGluR2-WT or mGluR2-C121A. The apparent difference in fluorescence intensity is attributed to the focal point, not the expression level of the receptors. (A) mGluR2-WT (B) mGluR2-C121A.
4.2. mGluR2 monomer-dimer equilibrium assessment by western blot

The western blot was performed on the membrane preparation of HEK-293 cells stably expressing either mGluR2-WT or mGluR2-C121A (Figure 4.3). The monomeric HA-tagged mGluR2 with mCitrine fluorescent protein after post-translational modification weighs about 140 kDa. Therefore, theoretical molecular weight of the homodimer would be about 280 kDa. The alpha-tubulin was detected as a loading control to ensure that equal amount of proteins were loaded.

As predicted, both monomeric and dimeric forms of the receptor were detected at the expected size on the wild type. The mutant mGluR2, on the other hand, had a significantly less dimeric form of the receptor compared to that of the wild type. The 140 kDa band intensities corresponding to the mGluR2 monomer was comparably similar between the wild type and the mutant.

For quantification of the bands, the intensity of these four bands (140 and 280 kDa of WT and C121A) were analyzed with densitometer, and the band intensity was plotted after normalized to the loading control (Figure 4.4). Similarly with the initial qualitative assessment, the band intensity for the mutant dimer was lower than that of the wild type while the band intensities corresponding to the monomer were comparably similar.
Figure 4.3 Western blot of the membrane preparation. mGluR2-WT (left) and mGluR2-C121A (right) show both monomeric and dimeric bands at 140 kDa and 280 kDa, respectively. There is significantly less dimeric population of the mutant relative to the wild type according to the estimation by the qualitative assessment on the WB. The monomeric band intensity of the wild type and mutant is comparable. The alpha-tubulin was detected as a loading control.
Figure 4.4 Quantified western blot band intensity by densitometer. Each intensity is normalized to the density counts of the alpha-tubulin. (A) the band pertinent to a monomeric mGluR2 receptor. The density counts are comparable. (B) the band pertinent to a dimeric mGluR2 receptor. The density counts are higher on mGluR2-WT.
4.3. Change in binding potential in $[^3]$HLY341495 saturation binding assay

The effect of the C121A mutation on binding characteristics was examined with radioligand binding assay. The membrane preparation was incubated with the radioactive mGluR2/3 antagonist, $[^3]$HLY341495, and the result was graphed with respect to the fmol of radioligand bound to milligram of total proteins (Figure 4.5). For this experiment, the HEK-293 cells stably expressing either mGluR2-WT or mGluR2-C121A were used as a source of receptors. Multiple clones of each cell line were used as replicates to confer statistical power. Binding curves produced from these clones were combined to generate Figure 4.5.

The $B_{\text{max}}$ refers to the total density of the receptors and is largely dependent on the expression level of the receptor in the cell. The $K_d$ is the radioligand equilibrium dissociation constant, which is the inverse of the receptor affinity to the ligand. Interestingly, both the $B_{\text{max}}$ and $K_d$ values were comparably similar as opposed to the initial hypothesis.

\[
BP = \frac{B_{\text{max}}}{K_D} = \text{receptor density} \times \text{affinity}
\]

Comprehensively, the BP (binding potential) coins these two different terms together to estimate a specific binding capacity of the receptor. As a result of the similar $B_{\text{max}}$ and $K_d$ values between the wild type and the mutant, the BP was not significantly affected by the C121A mutation.
Figure 4.5 Slightly lower binding potential of the mGluR2-C121A. The $[^3]H$LY341495 saturation binding curves are plotted. Due to the similar $B_{max}$ and $K_d$ values, the BP of the wild type and mutant is comparably similar. Calculated BP values for mGluR2-WT and mGluR2-C121A are 15497 and 12071, respectively. The experiments were performed in duplicate.
4.4. $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ functional assay indicates affected G-protein coupling

In order to assess the functional aspect of the mGluR2 upon C121A mutation, $G_i$ coupling of the receptor was examined by $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ functional binding assay. The radioactive $\text{GTP} \gamma \text{S}$ was incubated with the membrane preparation at different concentration of LY379268, a mGluR2/3 agonist, to allow the constitutive activation of the receptor. The vertical axis was reported as fold increases from the basal level of activation maintained without any agonist (Figure 4.6.1). Multiple clones of HEK-293 cells that either expresses mGluR-WT or mGluR2-C121A were used in this experiment to confer higher statistical power.

A combined binding curve from five different clones expressing mGluR2-C121A showed diminished $G_i$ coupling compared to that of the wild type (Figure 4.6.1). The EC50 is a value of the concentration of a drug at which half of the maximal response occurs. The EC50 value for mGluR2-WT is approximately 22 nM. As the mutant binding curve is slightly shifted to the right, the EC50 value has resultanty increased for mGluR2-C121A, which occurs around 40 nM. Each point of experiment with varying concentrations of LY379268 was quantitatively compared between the wild type and the mutant by unpaired t-test (Figure 4.6.2). The most significant statistical difference was observed at 100 nM concentration where the EC50 occurs.
Figure 4.6.1 Decreased Gi coupling of the mGluR2-C121A. The $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding curves are plotted. Diminished activation can be seen from mGluR2-C121A compared to the wild type. The curve is slightly shifted to the right responsible for the slightly increased EC50 value. The experiments were performed in triplicate.
Figure 4.6.2 Quantitative analysis of the $[^{35}\text{S}]\text{GTPyS}$ binding assay. Each point of LY379268 concentration was compared using unpaired t-test ($p < 0.05$). Statistical difference was began to be seen from 10 nM LY379268. The most significant difference between the wild type and the mutant was observed at 100 nM concentration of LY379268 where their EC50 values occur. ***$P < 0.001$, ****$P < 0.0001$. Data are means ± SEM of experiments performed in triplicate.
4.5. mGluR2 modulation in 5-HT$_{2A}$-dependent psychosis mouse model

The effect of functional mGluR2 was evaluated in mice that were evoked DOI-induced head twitch behavior. In the first experiment, the mGluR2/3 antagonist, LY341495 (3 mg/kg), was administered in mice before they were injected with DOI (0.5 mg/kg) to induce the HTR. The analyzed data were plotted to compare the HTR between the saline and LY341 group (Figure 4.7.1; Figure 4.7.2). The HTR of the saline group was maintained on the average of 20 head twitches in each 15 minutes blocks for 90 minutes. The HTR of the LY341 group was significantly enhanced after 30 minutes of the drug administration where the most difference can be seen in the first 45 minutes of DOI injection. Furthermore, the drug itself did not increase the HTR. The effect of the drug was seen only when 5-HT$_{2A}$-dependent HTR was induced by DOI. After 45 minutes since the DOI administration, the HTR started decreasing as the drugs were being washed out.

The following experiment was performed with the mGluR2/3 agonist, LY404039 (5 mg/kg). The previous experiments (Figure 4.9.1; Figure 4.9.2) indicated relatively short half-life of the drug, thus the drug incubation time was maintained only for 5 minutes before injecting mice with DOI (1 mg/kg). The analyzed data were plotted to compare the HTR between the saline and LY404 group (Figure 4.8.1; Figure 4.8.2). As the DOI used in this experiment was doubled, the average HTR was higher than that of LY341. For the LY404 group, the HTR was significantly attenuated in the first 30 minutes. After the active drug period, the head twitch counts continued to increase until they reached the comparable level as the saline group. Similar to the LY341 experiment, the LY404 itself did not decrease the head twitch counts.
Figure 4.7.1 Positive 5-HT$_{2A}$ modulation by mGluR2 deactivation. The initial 15 minutes of basal measurement served as habituation period. Either vehicle (saline) or drug (LY341495) was injected into mice after the habituation period. After 30 minutes from the time of injection, DOI was administered. HTR of animal was recorded throughout the experiment. Statistically significant multiple t-tests in all DOI groups ($p < 0.05$).
Figure 4.7.2 Positive 5-HT$_{2A}$ modulation by mGluR2 deactivation. The initial 15 minutes of basal measurement served as habituation period. The mice were injected with either saline or LY341495. Two-way ANOVA analysis shows statistically significant interactions between saline and LY341 groups ($F_{(8,234)} = 3.986; P = 0.0002$). $p < 0.05$, significant difference was observed between the period of DOI(15m) and DOI(75m) from the ANOVA and Sidak’s post hoc tests. ** $P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Notice statistical significance is lost after 75 minutes of DOI administration (n = 14 per each group).
Figure 4.8.1 Negative 5-HT2A modulation by mGluR2 activation. The initial 15 minutes of basal measurement served as habituation period. Either vehicle (saline) or drug (LY404039) was injected into mice after the habituation period. After 5 minutes from the time of injection, DOI was administered. HTR of animal was recorded throughout the experiment. Statistically significant multiple t-tests in first two DOI groups (p < 0.05).
Figure 4.8.2 Negative 5-HT2A modulation by mGluR2 activation. The initial 15 minutes of basal measurement served as habituation period. The mice were injected with either saline or LY404039. Two-way ANOVA analysis shows statistically significant interactions between saline and LY404 groups ($F(7,176) = 7.126; P < 0.0001$). $p < 0.05$, significant difference was observed between the period of DOI(15m) and DOI(30m) from the ANOVA and Sidak’s post hoc tests. ** $P < 0.01$, ****$P < 0.0001$. Notice statistical significance is lost after 30 minutes of DOI administration ($n = 12$ per each group).
Figure 4.9.1 LY404039 incubation for 30 minutes. The initial experiment with 30 minutes of drug incubation. Either vehicle (saline) or drug (LY404039) was injected into mice after the habituation period. After 30 minutes from the time of injection, DOI was administered. HTR of animal was recorded throughout the experiment. Notice the decrease in head twitch counts while the drug is still effective during the first 15 minutes of DOI administration (n = 6 per each group). Statistically significant multiple t-tests in only the first DOI groups (p < 0.05).
Figure 4.9.2 LY404039 incubation for 30 minutes. The initial 15 minutes of basal measurement served as habituation period. The mice were injected with either saline or LY404039. Two-way ANOVA analysis shows statistically significant interactions between saline and LY404 groups ($F_{(8,90)} = 2.152; P = 0.0387$). $p < 0.05$, significant difference was still observed at the time period of DOI(15m) from the ANOVA and Sidak’s post hoc tests. Most of the statistical power is lost compared to the subsequent experiment with 5 minutes drug injection (Figure 4.8.2). *$P < 0.05$. Notice statistical significance is immediately lost after 15 minutes of DOI administration ($n = 6$ per each group).
Chapter 5. General Discussion

In order to elucidate the functional role of C^{121} in homodimeric mGluR2, a point mutation was introduced in the human mGluR2 gene to replace cysteine with alanine at the corresponding location. The mGluR2 carries the HA-tag at N-terminal end with the mCitrine fluorescent protein conjugated at the C-terminal end of this protein. These two elements were engineered into its gene primarily for visualization purposes by serving as epitopes for antibody, which were used on immunofluorescence microscopy and western blot protocols. Furthermore, the first 54 bps of the gene, which translates the human mGluR2 signal peptide, was replaced with the rat mGluR5 signal peptide sequence, because the signal peptide cleavage during translocation into endoplasmic reticulum (ER) also removed the HA-tag in the process. Therefore, the rat mGluR5 signal peptide sequence was used instead, which would still localize the mGluR2 into the ER while it does not interfere with the functionality of the receptor (Bhave, 2003).

Next, sequence verified mGluR2 recombinant genes were transiently transfected into the HEK-293 cells. As the previous experiment (not shown) with 48-hour incubation showed comparable expression level of 24-hour incubation, all subsequent experiments were carried out with a 24-hour incubation period. Also, the transfection protocol was optimized prior to the experiments (Figure 4.1.1). Since the transfection with PEI using 10 ug of plasmids without antibiotics yielded highest transfection efficiency, such specifics were used throughout the experiment. On a side note, the PEI was selected over Lipofectamine 2000 due to the economic advantage and Lipofectamine’s sensitivity to plasmid to Lipofectamine ratio, which attributes to
the transfection failure when 12 ug plasmids were used without compensating for the vehicle reagent (Figure 4.1.1, F).

The effect of C121A mutation on the receptor expression and subcellular localization was explored by using immunofluorescence microscopy technique. The cells grown on the coverslips were also treated with a chemical detergent to perforate the cell membrane. The anti-HA tag antibody was used to detect the receptors trafficked to the cell surface where the antibody was only accessible as long as the cell did not lose its structural integrity. As it can be seen from the immunofluorescence images (Figure 4.1), the fluorescence from the antibody was detected on the non-permeabilized cell surface, which suggests that the mutation did not compromise receptor’s ability to traffic to the cell membrane. However, the effect of this mutation on trafficking should not be disregarded as it can partially affect the process. When the cells were permeabilized with the detergent, the signal from antibody accurately co-localized with mCitrine fluorescence indicating that there is subcellular population of the intact receptors. For that matter, the mutation seems to subcellularly accumulate receptors in the cells. One hypothesis is that the lack of covalent stability lowered the efficiency of dimerization in that it increased the number of monomeric receptors in the cells, which were unable to traffic to the cell surface. However, this idea is only reasonable if there is an evidence that dimerization occurs prior to reaching the cell surface. Therefore, the next step would be to produce the receptors that are incapable of dimerizing to see if monomers traffic to the plasma membrane of the cell. If these monomers fail to exist at the surface level, it will further support the hypothesis that C121A affects the homodimerization of mGluR2.
To bolster the qualitative assessment by immunofluorescence microscopy, the western blot technique was used to quantitatively analyze mGluR2 monomer-dimer equilibrium (Figure 4.4). Here, previously generated stable cell lines that constitutively expresses either mGluR2-WT or mGluR2-C121A were harvested for the membrane preparation, which was chemically reduced before running in the SDS-PAGE. As a side note, the membrane samples were not heat treated due to the formation of aggregate falsely contributing to increased dimer population. Even after these harsh treatments, it is surprising to see the presence of significant amount of dimer in the western blot. It may be due to several other cysteine residues from CRD and LBD that could potentially contribute to the stability of dimers (Muto, 2007; Niswender, 2010). Regardless, a distinctive difference in the monomer-dimer equilibrium between these two receptors was visible (Figure 4.3). The 140 kDa band is approximately the molecular weight of the post-translationally modified mGluR2-mCitrine. From the quantitative analysis by densitometry, the monomeric band intensity of both the wild type and the C121A mutant was comparably similar indicating that expression level of mGluR2 was not affected by possible alteration in trafficking. On the contrary, the 280 kDa band, that is pertinent to the dimeric population of mGluR2, was affected by the mutation. As expected, the formation of the dimer was significantly reduced by the C121A mutation as it was seen from the western blot image. This finding can serve as an additional evidence that C121A is involved in the dimerization process and structural stability of homodimer.

Interestingly, there were two bands corresponding to the MW of mCitrine (not shown). This can be a possible indication that there is a population of the receptor without the mCitrine, in which case explains the smearing of the band as different combinations of heteromer are
possible. It is also relevant to note that the intensities of the loading control were comparable. This further validates the result produced in the western blot.

Subsequently, the effect of C121A mutation on ligand binding property was assessed by radioligand saturation binding assay with the $[^3H]LY341495$. This substrate is a specific orthosteric antagonist for group II mGluRs, which is known to increase the effect of 5-HT$_{2A}$-dependent hallucinogenic drugs in a mouse model (Gewirtz, 2000). The ability of binding mGluR2 orthosteric site and its high affinity to the receptor make $[^3H]LY341495$ a great candidate for receptor quantification and characterization of binding capacity. As for the receptor source, the cell lines that stably express either mGluR2-WT or mGluR2-C121A were generated as previous results from the transiently transfected cells were inconsistent due to the varying transfection efficiency for each experiment (not shown). Several binding curves generated from different cell lines were combined to produce two averaged binding curves for comparison.

Surprisingly, binding properties and pharmacokinetics of the receptor was not affected by C121A mutation. Furthermore, the $B_{\text{max}}$ and $K_d$ values are comparably similar although calculated BP values indicates slightly higher binding potentials of wild type receptor. This suggests that the mutation changed binding affinity to a certain degree, however, it is arguable if this effect is functionally significant. In spite of these changes, the binding profile does not seem to be affected for the most part.

In order to investigate the functional consequences of C121A mutation, $[^{35}S]$GTP$_\gamma$S binding assay was conducted to assess the heterotrimeric G-protein coupling of mGluR2. A specific ratio of radioactive GTP$_\gamma$S and GDP was incubated with the membrane preparation to allow the constitutive activation of the receptor. To activate the receptor, a specific orthosteric...
agonist for group II mGluRs known as LY379268 was used to generate dose-response binding curves.

While the mutation did not have much impact on pharmacokinetics of mGluR2 as it was demonstrated from $[^3H]LY341495$ saturation binding assay, it is interesting to see that $G_i$ coupling has significantly diminished in mGluR2-C121A. It is possibly due to the need for dimerization in taking activatable conformation. This suggests that binding characteristics of mGluR2 was not sufficiently influenced by the mutation whereas the dimerization property was negatively altered to ultimately reduce G-protein coupling affecting the function of the receptor.

Lastly, the mGluR2 modulation of the 5-HT$_{2A}$-dependent hallucination was revisited in the context of the head twitch response. This experiment was done as a partial evidence for the overall scheme of the crosstalk between 5-HT$_{2A}$ and mGluR2, which will help elucidate the heteromerization mechanism. To achieve that, C57BL/6 mice are injected with either LY341495 or LY404039 that are mGluR2/3 antagonist and agonist, respectively. These pre-treatments were followed by DOI administration to induce the HTR.

As expected, the LY341495 visibly increased HTR and its effects lasted up to 75 minutes from the DOI injection until the statistical significance of HTR difference was lost. It is important to note that the antagonist alone did not alter the head twitch counts. It increased the counts only in conjunction with the DOI injection despite the low dosage of DOI. These results demonstrate the ability of LY341495 to potentiate the actions of 5-HT$_{2A}$ by inactivating mGluR2. Subsequent cross-group experiments resulted in very similar outcomes, which were then combined with the initial experimental data to produce the reported graphic plot (Figure 4.7.1).
Followed by the antagonist experiments, the effect of mGluR2 activation on 5-HT$_{2A}$-dependent HTR was investigated using LY404039. Here, the mGluR2 agonist noticeably and immediately decreased head twitch counts for 30 minutes, which the effect of the drug seems to have expired after that time point. Similar to the antagonist experiments, the drug was not responsive to the basal level head twitch counts, and it rather directly modulated the behavioral effect of 5-HT$_{2A}$ (Figure 4.8.1). In this experiment, the incubation time for LY404039 was limited to only 5 minutes as the previous experiments with longer incubation period resulted in less statistical power due to a short half-life of the drug (Figure 4.9.1).

With the 5-HT$_{2A}$-dependent behavior modulation by either mGluR2 agonist or antagonist, it was successfully shown here that mGluR2 affects the effect of 5-HT$_{2A}$. Also, these drugs specifically targeted the actions of 5-HT$_{2A}$ without changing the basal HTR. In other words, the antagonist potentiated the 5-HT$_{2A}$ and the agonist interfered with the signaling by 5-HT$_{2A}$. While these animal studies have shown the interaction between 5-HT$_{2A}$ and mGluR2, these experiments alone do not provide a sufficient evidence for either heteromerization or 5-HT$_{2A}$-mGluR2 crosstalk as they could work independently. However, other studies have already reported biochemical and electrophysiological corroborations for the existence of 5-HT$_{2A}$-mGluR2 heteromer, thus these behavioral data support the claims for this relatively novel idea (Albizu, 2010; Gonzalez-Maeso, 2008; Levitz, 2016; Xue, 2015).

Throughout the thesis, continuous efforts to elucidate the role of C121A mutation on mGluR2 were expressed from the perspective of biochemistry and molecular biology. The absence of this conserved cysteine residue affected dimerization property leading to more subcellularly populated monomeric mGluR2 as it was shown by immunofluorescence
microscopy while disturbed dimer formation was observed from the western blot. From the [³H]LY341495 saturation binding assay, the mutation did not seemed to affect binding characteristics of mGluR2 as both Bmax and Kd values were comparably similar. On the other hand, Gi coupling was diminished on mGluR2-C121A as it was shown in the [³⁵S]GTPγS binding assay suggesting a potential adverse impact the mutation has on proper functioning of mGluR2. Finally, 5-HT2A-dependent increase or decrease in HTR by mGluR2 modulation in a mouse model further supported the existence of 5-HT2A-mGluR2 crosstalk. As a future direction, it would be interesting to perform follow-up studies on heteromerization of 5-HT2A with this particular mutant receptor to compare the receptors interaction and crosstalk efficiency.

Collectively, the C¹²¹ plays a notable part in the formation and maintenance of mGluR2 homodimer, and therefore calls for further investigation on subcellular localization, dimerization, binding affinity, and functionality of mGluR2-C121A as it could shed light on the specifics of the mGluR2 signal mechanism which may involve 5-HT2A and perhaps other receptors as well.
References


Vita

Education

- **The Virginia Commonwealth University** – Richmond, VA (GPA: 4.0/4.0) August 2018
  - Masters of Science, **Physiology and Biophysics**

- **The Virginia Commonwealth University** – Richmond, VA (GPA: 4.0/4.0) May 2017
  - Certificate, **Premedical Graduate Health Science Certificate Program (CERT)**

- **The Pennsylvania State University** – University Park, PA (GPA: 3.5/4.0) May 2013
  - Bachelor of Science, **Biochemistry and Molecular Biology - Biochemistry option**
  - Bachelor of Science, **Immunology and Infectious Diseases**
  - Minor in **Microbiology**

Manuscripts in preparation

- Hideshima KS, Hojati A, Saunders JM, On DM, de la Fuente Revenga M, **Shin JM**, Sanchez-Gonzalez A, Dunn CM, Pais AB, Pais AC, Miles MF, Wolstenholme JT, González-Maeso J. Role of mGlu2 in the 5-HT2A receptor-dependent antipsychotic activity of clozapine in mice (manuscript under final review at *Psychopharmacology*)

- de la Fuente Revenga M, **Shin JM**, Hideshima KS, González-Maeso J. Fully-automated rapid head movement detection system for the screening of antipsychotic drugs in vivo (manuscript submitted to *Scientific Methods*)

- Toneatti R, **Shin JM**, Shah UH, Saunders JM, Mayer CR, Lopez-Gimenez JF, Janssen WG, Benson DL, Conway DE, González-Maeso J. Serotonin 5-HT2A receptor regulates mGlu2 receptor cell surface delivery and endocytosis via GPCR heteromerization (manuscript in preparation)

- Shah UH, Gaitonde SA, **Shin JM**, Toneatti R, González-Maeso J. Genetically encoded photocross-linkers locate the heteromeric interface in a serotonin GPCR heteromer (work in progress)

- **Jong M. Shin** (...) Javier Gonzalez-Maeso. Disruption of mGluR2 homodimerization affects receptor trafficking and function (work in progress)

Posters and Presentation

1. **Jong M. Shin**, Shagufta Khan, Raj Kumar, Jun Ling. The contribution of c-MYC to glucocorticoid regulated breast cancer cell proliferation. Geisinger Commonwealth School of Medicine, PA, July 25, 2012

Experience

- **Emergency Department Medical Scribe** – August 2017 to Present  
  *Bon Secours Richmond Emergency Physicians, Inc.* - Richmond, VA  
  - Documented the physician dictated patient history, physical examination findings and procedures as performed by the physician.  
  - Documented the results of laboratory and radiographic studies as dictated by the physician.

- **Teaching Assistance for undergraduate physiology lab** – August 2017 to May 2018  
  *The Virginia Commonwealth University, Physiology and Biophysics Department* - Richmond, VA  
  - Provided physiology lectures to 160 undergraduate students for two semesters  
  - Provided guidance to students for fluid learning of complex concept of physiology integrated with physical experiments

- **Graduate Student under Dr. Javier Gonzalez-Maeso** - June 2017 to Present  
  *The Virginia Commonwealth University, Physiology and Biophysics Department* - Richmond, VA  
  - Investigate the effect of C121A mutation of mGluR2 receptor on homodimer formation  
  - Perform binding assays using different radioligands to study membrane receptor expression and functionality of GPCR relevant to heteromer formation involved in schizophrenia  
  - Conduct behavioral research on C57BL/6 mice to study the potentiation of LY341495 to DOI head twitch response effect

- **Laboratory Specialist under Dr. Scott Zeitlin** - June 2015 to Oct 2015  
  *University of Virginia School of Medicine, Neuroscience Department* - Charlottesville, VA  
  - Independently managed over 200 mouse cages at a time and produced various genetic crosses of mouse colonies to be used on histologic and behavioral studies  
  - Performed genotypic analysis of the transgenic mice that expresses human huntingtin's gene and ensured positive gene expression controlled by IPTG administration

- **Business Coordinator** - May 2013 to May 2015  
  *Mansoo Health* - Annandale, VA  
  - Maintained fluent flow of the furniture retail business and provided technical support  
  - Provided over 700 translations in both English and Korean

- **Undergraduate Researcher under Dr. Katsuhiko Murakami** - May 2011 to May 2013  
  *Pennsylvania State University, Biochemistry and Molecular Biology Department* – State College, PA  
  - Worked over 20 hours per week participating in numerous RNA polymerase projects  
  - Performed purification of DNA/RNA/proteins and other various biochemical techniques relevant for X-crystallographic structural analysis

- **Research Intern under Dr. Jun Ling** - June 2012 to Aug 2012  
  *Geisinger Commonwealth School of Medicine* – Scranton, PA  
  - Researched the effect of c-Myc inhibition on different human breast cancer cell lines  
  - Attended a number of medical school classes and medical science seminars
- **U.S. Navy Orthopedics Technician** - June 2008 to June 2010  
  *U.S. Yokosuka Naval Hospital* - Yokosuka, Japan  
  - Provided over 1,000 patients with casts and splints during their clinic visits, emergency room visits, and surgical procedures.

- **U.S. Navy Gas Turbine Systems Electrician** - November 2006 to June 2008  
  *USS John S. McCain* - Yokosuka, Japan  
  - Operated, repaired, and performed maintenance of gas turbine engine, main propulsion machinery and control systems.

---

### Awards and Recognition

- **The Physiology and Biophysics Department Certificates of Recognition (2018)**
- **Phi Kappa Phi Honor Society Academic Achievement Nominee (2018)**
- **Undergraduate Exhibition in Life Science Division Grant Recipient (2011)**  
  - Awarded $2,000 research grant for entrance proposal: *Structure of the Transcriptional Elongation Complex of Human Mitochondrial RNA Polymerase*
- **George and Elizabeth Smollett Sperling Trustee Scholarship (2011)**
- **Bunton-Waller Scholarship (2011)**

---

### Honors

- **Phi Kappa Phi Honor Society (Since 2017)**
- **Pennsylvania State University Dean’s List (2011 – 2013)**

---

### Skills

- **Laboratory Techniques**
  - Mammalian and bacterial cell culture
  - Nucleic acid extraction
  - PCR
  - Genotypic analysis of mammalian tissue biopsy
  - Molecular cloning
  - Familiar with Protein purification with ion-exchange and affinity column
  - Familiar with protein crystallization
- **Computer Skills**
  - Java, Visual Basic, MATLAB, Prism, ZEISS ZEN, HTML, Microsoft Office, Adobe Photoshop CS, CLC sequence viewer
- **Language Skills**
  - Korean, English, and Japanese

---

65