2018

Chronic Clozapine Treatment Impairs Functional Activation of Metabotropic Glutamate Receptor 2 via an HDAC2-depedent Mechanism

Travis M. Cuddy

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University.

by

Travis M. Cuddy
B.S., Biochemistry & Molecular Biology, University of Richmond, 2015.

Director: Dr. Javier González-Maeso
Associate Professor, Department of Physiology and Biophysics

Virginia Commonwealth University
Richmond, Virginia
April 2018

Manuscripts Resulting from this Work

Co-authored Manuscripts Under Review

de la Fuente Revenga M, Ibi D, **Cuddy T**, Toneatti R, Kurita M, Ijaz M, Miles MF, Wolstenholme JT, and González-Maeso J.

"Clozapine treatment restrains via HDAC2 the performance of mGlu2/3 agonism in a rodent model of antipsychotic activity."

Under review; revised article soon to be submitted to ***Neuropsychopharmacology***

de la Fuente Revenga M, Ibi D, Saunders JM, **Cuddy T**, Ijaz MK, Toneatti R, Kurita M, Holloway T, Shen L, Seto J, Dozmorov MG, and González-Maeso J.

"HDAC2-dependent antipsychotic-like effect of chronic treatment with the HDAC inhibitor SAHA in mice."

Under review; revised article submitted to ***Neuropharmacology***

First Author Manuscripts

Cuddy *et al*. "Characterization of functional G protein coupling induced by the mGlu2/3 receptor agonist LY379268 in mouse frontal cortex."

In progress.

Acknowledgement

I would like to thank my parents, June and James Cuddy, as well as my grandfather, Michael Cuddy, for their unfailing love and support throughout all of my academic endeavors. I would like to thank Dr. Mario de la Fuente Revenga for his willingness to teach me everything I needed to know in order to complete this project on my own. I would like to thank all of the members of my laboratory for their part in creating a lively, collaborative, and productive environment that helped me to succeed. Last, but certainly not least, I would like to thank Dr. Javier González-Maeso, whose invaluable mentorship helped me to realize my potential in the laboratory.

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List of Abbreviations

[^{35}S]GTPγS	Guanosine triphosphate, radiolabeled on the gamma phosphate group with sulfur-35
5-HT$_{2A}$	5-Hydroxytryptamine (serotonin) receptor 2A
CNS	Central nervous system
DRD2	Dopamine receptor D2
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
HDAC2	Histone deacetylase 2
mGluR2	Metabotropic glutamate receptor subtype 2
mGluR3	Metabotropic glutamate receptor subtype 3
MK-801	Dizocilpine
NAM	Negative allosteric modulator
NIMH	National Institute of Mental Health
NMDA	*N*-methyl-D-aspartate
PAM	Positive allosteric modulator
PANSS	Positive and Negative Syndrome Scale
PCP	Phencyclidine
SAHA	Suberanilohydroxamic acid (a broad-spectrum HDAC inhibitor)
SOC	Standard of care

Abstract

Chronic Clozapine Treatment Impairs Functional Activation of Metabotropic Glutamate Receptor 2 via an HDAC2-depedent Mechanism

By Travis M. Cuddy, B.S.

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University.

Virginia Commonwealth University, 2018.

Major Director: Dr. Javier González-Maeso, Associate Professor,
Department of Physiology and Biophysics

Schizophrenia is a chronic mental disorder affecting millions worldwide. It has no known cure. Current pharmaceutical treatments have shown efficacy in only one of the three symptom clusters of schizophrenia, providing little or no benefit in the other two. Furthermore, the current standard-of-care drugs, known as atypical antipsychotics, carry risks of severe side effects affecting multiple body systems. Most patients opt to discontinue drug therapy within two years of initiation due to lack of efficacy and/or preponderance of adverse effects. Previous findings have shown that chronic usage of atypical antipsychotics causes a 5-HT_{2A}-dependent upregulation of histone deacetylase 2 (HDAC2), which in turn leads to downregulation of metabotropic glutamate receptor 2 (mGluR2), a G protein-coupled receptor with an important role in synaptic plasticity. The

present study aims to characterize the extent to which this downregulation leads to specific functional outcomes, and in doing so, may help identify new targets for more effective treatment of schizophrenia.

Chapter 1: Introduction

1.1: Schizophrenia and its Treatment

Schizophrenia is a chronic, severe, and debilitating mental disorder that impacts how a person thinks, feels, and behaves.[1] The disorder carries considerable societal costs[2] and afflicts approximately 1% of the world's population.[3] Symptoms most often appear in late adolescence and early adulthood, and are grouped into three main classes: positive symptoms (e.g. hallucinations, delusions), negative symptoms (e.g. flattened affect, anhedonia), and cognitive deficits (e.g. poor executive function, problems with working memory).[1] Despite the fact that the precise causes of the disorder remain unknown, there is a consensus that schizophrenia is multifactorial in origin, arising from interactions between genetic, environmental, psychological, and social factors.[4]

Pharmacological treatment for schizophrenia consists primarily of a class of drugs known as antipsychotics. While effective against positive symptoms of the disorder in 30-40% of patients,[5] antipsychotics provide limited, if any, relief from the negative and cognitive symptoms,[6] which are predictors of functional disability.[7] Persons with schizophrenia have only a 5-10% chance of making a full recovery regardless of antipsychotic use.[5] In addition to these considerable shortcomings, antipsychotics also carry a vast array of side effects, some of which are severe.

Consequently, rates of non-adherence to medication are high in this population – as many as 75% of patients stop treatment within two years of initiating therapy.[8] Without doubts, treatment of schizophrenia is an area where current clinical needs remain woefully unmet.

The extent of this problem is perhaps best illustrated by the story of the antipsychotic drug clozapine, which was developed in the 1960s. Today, over half of a century later, it remains the only drug proven to be effective for treatment-resistant schizophrenia.[8] Despite a lowered incidence of extrapyramidal motor symptoms, clozapine still carries risk for many adverse effects,[9] with risks of agranulocytosis, considerable weight gain, diabetes, metabolic syndrome, bowel obstruction, and other serious problems.[10] Dishearteningly, in the many years since the development of clozapine, all attempts to create a compound that retains its unmatched efficacy – yet sheds its many adverse effects – have failed.

1.2: Typical Antipsychotics

The earliest hypotheses of schizophrenia emerged from the discovery of the antipsychotic action of chlorpromazine and the later characterization of its target, the dopamine D2 receptor (DRD2). The foundation of the dopamine hypothesis of schizophrenia can be traced back to the 1970s, when reports that the known antipsychotic drugs were antagonists at DRD2 and that clinical efficacy of these drugs was strongly correlated to their affinity for the striatal DRD2. Further support for this hypothesis was derived from observations that high amounts of amphetamine, a

dopamine transporter substrate, can induce psychotic symptoms, as well as reports that reserpine, a drug that depletes monoamine neurotransmitter levels in synapses, was capable of reducing such symptoms.[8] Until recently, the prevailing notion was that dysfunctional dopaminergic activity in forebrain regions was largely responsible for schizophrenia.[11] The earlier antipsychotic drugs (e.g. chlorpromazine, haloperidol), now referred to as "typical antipsychotics," are potent DRD2 antagonists, though they exert additional effects on a variety of targets. These drugs have a high incidence of extrapyramidal motor side effects, such as tardive dyskinesia, which complicates their use.

1.3: Atypical Antipsychotics

More recently, research regarding the molecular underpinnings of schizophrenia has focused on other neurotransmission systems, such as serotonin and glutamate. Whereas the typical antipsychotic drugs are DRD2 antagonists, newer drugs also exhibit potent antagonism at the serotonin receptor 2A (5-HT_{2A}), generally in addition to slightly lowered affinity for DRD2. These drugs, referred to as "atypical" antipsychotics, have now become first-line treatments for patients with schizophrenia.[12] Compounds in this class include clozapine, risperidone, and olanzapine. Their main advantage over the older, typical antipsychotics is a lowered incidence of extrapyramidal motor side effects. However, as previously mentioned, the atypical medications are not without their own risk of serious adverse effects, and only address certain symptoms. Drugs for

schizophrenia are in need of improvement, but additional information on mechanism(s) of the disorder is required to guide new drug development.

1.4: Glutamatergic Dysfunction and New Drug Development

Recent discoveries concerning glutamatergic dysfunction in schizophrenia have buttressed attempts to develop a newer class of antipsychotic drugs. Efforts in this area in particular were made by Eli Lilly & Co., who created and incrementally improved a novel class of compounds – the group II metabotropic glutamate receptor agonists – that showed promise for antipsychotic utility, culminating in LY2140023 (pomaglumetad methionil). While preclinical[13] and early clinical[14] studies were encouraging in this regard, ensuing clinical trials[15-18] were not, as they failed to demonstrate efficacy of LY2140023 in treatment of schizophrenia. Ultimately, Lilly abandoned phase III development in 2012.[19]

During the past six years, new research findings[20-22] have revealed more information about how metabotropic glutamate receptor 2 (mGluR2), one of the targets of the Lilly-developed agonists, is regulated. In elucidating the nature of the mechanism that underlies this regulation as well as its functional consequences, these works, in conjunction with the present study, might offer a possible explanation as to why the newer drug was only effective in the patient population used in the earliest clinical study, and not in other patients. In the years since the trials' completion, multiple authors[23,24] have raised the concern that patient selection, which was done without knowledge of how prior treatment with atypical antipsychotics might affect the potential of newer

drugs, could be at least partially responsible for the new drug's failure to show efficacy in later trials. Progress in this area will further enrich knowledge of schizophrenia and its treatment at the molecular level, thereby aiding in attempts to develop new and improved treatment strategies, an urgent clinical need.

1.5: G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are a large and diverse family of integral membrane proteins found in eukaryotes. GPCRs achieve signal transduction across membrane barriers by coupling extracellular ligand binding to the initiation of intracellular signaling cascades. Their structure is well-suited to carry out this function, consisting of an extracellular N-terminus, an intracellular C-terminus, and seven transmembrane domains that are held together by both intra- and extracellular loops (Fig. 1). Intracellular portions of the GPCR are responsible for coupling to heterotrimeric G proteins, consisting of α, β, and γ subunits (Fig. 1). In response to ligand binding events occurring on the extracellular aspect of the GPCR, the G protein undergoes a conformational change and becomes activated. As a result, it initiates intracellular signaling pathways that can regulate a wide array of downstream effector molecules (e.g. ion channels, enzymes, and transcription factors), ultimately leading to a biological response (Fig. 1). There are multiple classes of GPCRs, and members of different classes exhibit some differences in structure and function. Class C GPCRs, such as the metabotropic glutamate receptors, contain the ligand-binding site within the extracellular N-terminus, which is distinctly enlarged among members of this class.[25]

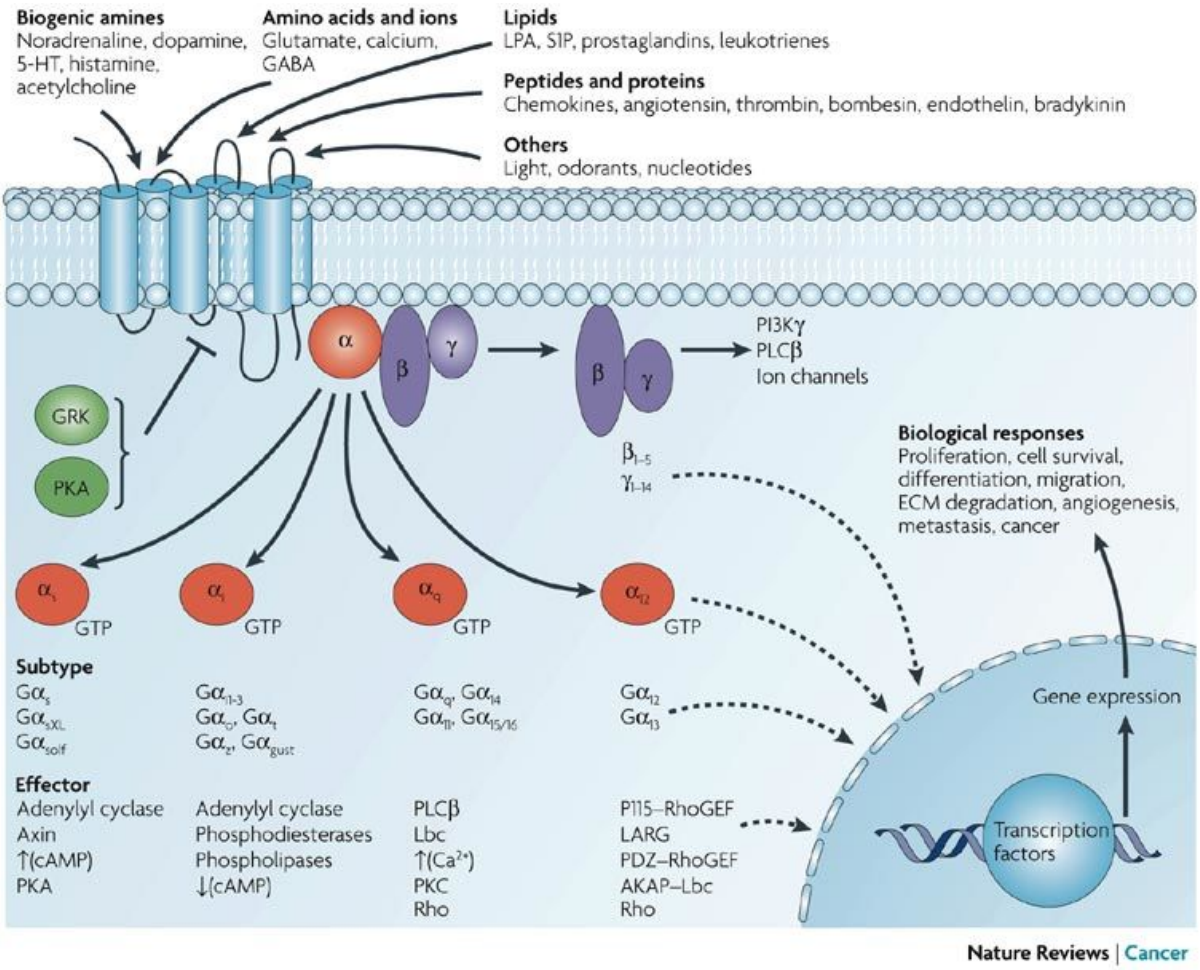


Figure 1. General structure and scheme of GPCRs. Ligands (i.e. biogenic amines, amino acids, ions, lipids, peptides, proteins, etc.) bind to extracellular portions of the GPCR as a means to reach targets on the intracellular side of the membrane, in the cytoplasm, and in the nucleus. The GPCR is coupled to heterotrimeric G proteins, which are made up of the subunits α, β, and γ. Upon activation by a ligand, the receptor undergoes a conformational change that results in the replacement of GDP bound to the α subunit with GTP, causing the α subunit to dissociate from the other subunits. There are four families of Gα subunits, each of which are associated with distinct signaling cascades. Functional activation of the GPCR has many important regulatory functions in an array of intracellular signaling networks. As such, disruption of normal functional activation can have far-reaching implications for the cell. Image source: Dorsam and Gutkind, 2007[26]. Reproduced with permission.

1.6: Histone Deacetylases

Histone deacetylases (HDACs) are widely-expressed enzymes that perform important epigenetic regulatory functions. Generally, HDACs deacetylate (i.e. catalyze the removal of an acetyl group from) lysine residues on histones. This alters the conformation of the histone N-terminal tails, which has the ultimate consequence of pulling the histones and their wound DNA more tightly together into an increasingly condensed state. Less-accessible chromatin correlates with reduced transcription and/or silencing of genes. (Fig. 2) Conversely, chromatin is rendered more “open” to transcriptional machinery by the opposing process of acetylation, performed by histone acetyltransferases. Normally, there is a carefully-controlled balance between deacetylation and acetylation, but this balance can be perturbed by numerous insults such as stress and disease (Fig. 2). Resultant dysregulation of transcription can lead to a variety of outcomes depending on what genes are affected. In neurons, normal expression of many genes related to synaptic plasticity is compromised by excessive HDAC2 activity. Drugs that inhibit HDACs may be able to restore a normal balance between transcriptional activation and lack thereof (Fig. 2), and are currently being explored for possible benefits in a number of diseases and conditions.

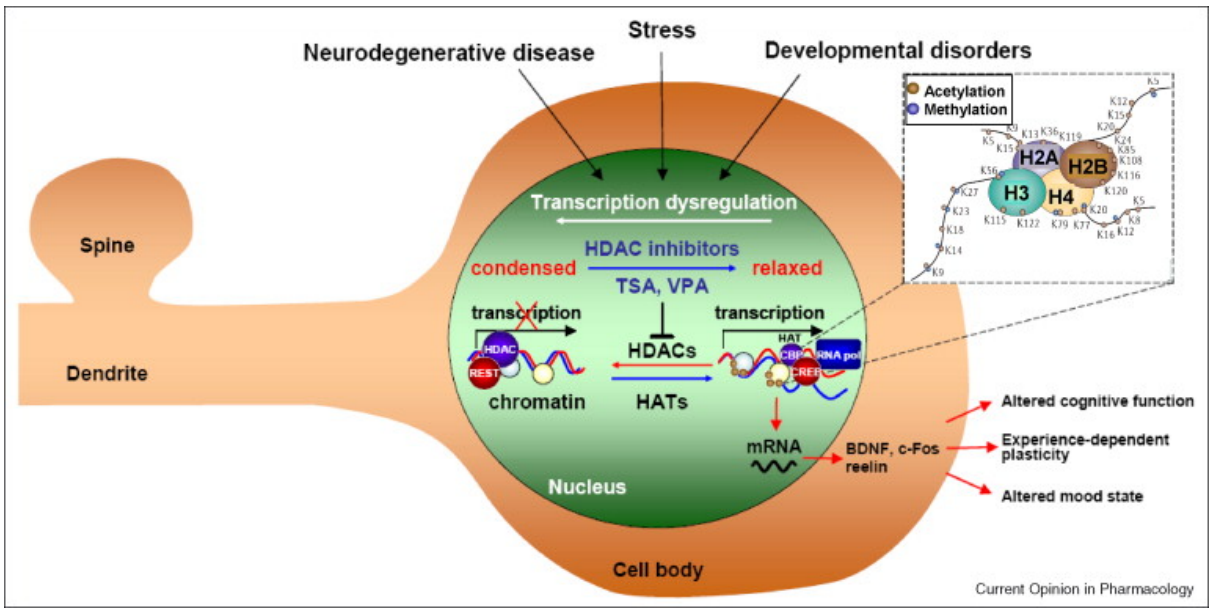


Figure 2. The opposing processes of histone acetylation and deacetylation. Shown here in a neuron, maintenance of the balance between histone acetylation and deacetylation is critical to ensure proper regulation of transcription in the cell. When this balance is disrupted, the cell is less able to control transcription of many genes important in cognitive function, synaptic plasticity, and mood state. HDAC inhibitors can be used to try and rectify imbalances resulting from excessive deacetylation of histones. Image source: Abel and Zukin, 2008.[27] Reproduced with permission.

While multiple HDACs exist, Histone deacetylase 2 (HDAC2) is of particular importance in the context of schizophrenia. Briefly, it has repeatedly been shown[20,28] that chronic treatment with atypical antipsychotics (e.g. risperidone, clozapine) causes an epigenetically-mediated downregulation of mGluR2 in the human and mouse frontal cortex in a 5-HT_{2A}-dependent manner, and that HDAC2 is a main effector in this process.

1.7: Rationale for This Study

As mentioned earlier, mGluR2 is a G protein-coupled receptor (GPCR), a class of proteins that accomplish signal transduction across a cellular membrane by coupling cell surface receptor binding with intracellular activation of a signaling cascade. This

characteristic makes mGluR2 amenable to certain modes of study. One way to gauge the extent to which mGluR2 downregulation by HDAC2 translates into functional outcomes is to measure the level to which mGluR2 couples extracellular ligand binding to initiation of intracellular signaling cascade (i.e. functional activation). This can be achieved in mouse brain samples by stimulating the mGlu2 receptors in the presence of a hydrolysis-resistant radiolabeled nucleotide that participates in the process of GPCR signal transduction.

While chronic clozapine treatment is known to decrease expression of mGluR2 in the frontal cortex via HDAC2 in a 5-HT_{2A}-dependent manner, the extent to which this action affects the function of mGluR2 has not been characterized. For this reason, the current study aims to assess the specific contribution of HDAC2 to loss of function of mGluR2 at the level of its G protein. As such, mice were treated chronically with clozapine in order to induce the HDAC2-mediated downregulation of mGluR2. Half of the mice had conditional knockouts of HDAC2 in cortical pyramidal neurons, whereas the other half had natively functional HDAC2 in those neurons. Upon completion of treatment, frontal cortex brain samples were assessed on the basis of functional activation by agonist-stimulated [^{35}S]GTPγS binding assay, and results compared across treatment (i.e. clozapine or saline) and genotype groups.

Chapter 2: Background

2.1: Chlorpromazine and the Dopamine Hypothesis of Schizophrenia

The earliest ideas about the underlying mechanisms of schizophrenia can be traced back to the development of the phenothiazine derivative chlorpromazine (Fig. 3) in the 1950s. A few years prior, in the late 1940s, French scientists at Rhône-Poulenc laboratories were investigating a series of substituted phenothiazine derivatives for many effects, one of which was antihistaminergic activity. These efforts led to the discovery of two closely-related and historically important drugs: 1) promethazine, a potent antihistamine that is still widely used today, and 2) chlorpromazine, another potent

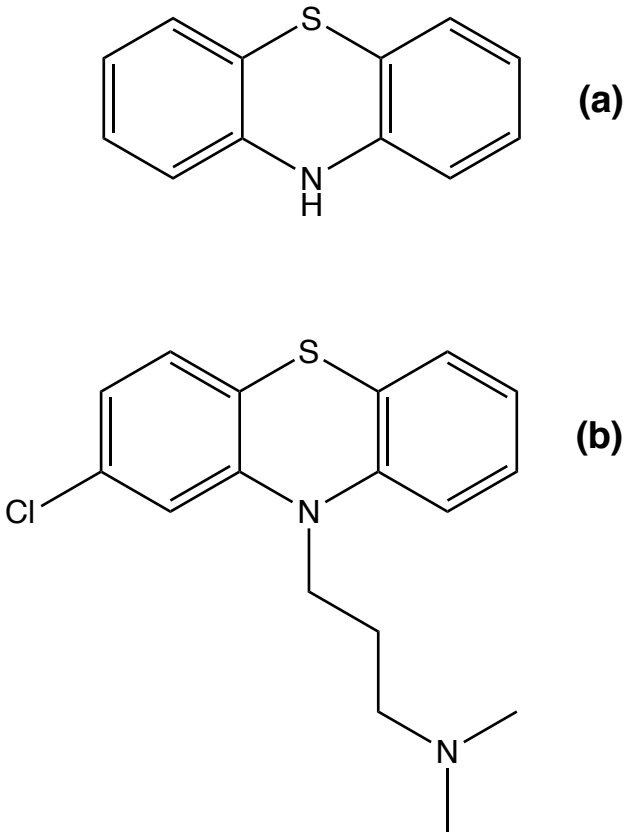


Figure 3. Phenothiazine and chlorpromazine. Chemical structures of phenothiazine (a) and its derivative chlorpromazine (b) are shown. Structure diagrams were produced in ChemDraw Professional version 16.0.

antihistamine whose additional characteristics, once found, would rapidly change the field of clinical psychiatry forever.[29]

In 1951, while attempting to harness the sedative effects of promethazine and chlorpromazine for use as surgical anesthetics, French surgeon Henri Laborit observed that patients given chlorpromazine before surgery not only became sedated but also displayed much less anxiety.[29,30] Believing that these effects might be useful in a psychiatric setting, Laborit gave the compound to psychiatrists working in psychiatric facilities in Paris,[29] where it was soon found to reduce symptoms of psychosis in a 1952 trial.[31] Later that year, Rhône-Poulenc approached three American pharmaceutical companies about the possibility of a licensing agreement to market chlorpromazine in the United States. After being turned down twice, Rhône-Poulenc reached a licensing agreement with U.S. company Smith, Kline & French (SK&F) in 1952. Extensive testing was performed, and by 1954, SK&F was marketing chlorpromazine as Thorazine in the United States.[32]

The introduction of chlorpromazine had a profound effect on the practice of clinical psychiatry in the United States. Until that time, American psychiatrists were not using drugs to treat mental illness. Instead, they relied almost exclusively on shock therapies and/or various forms of psychotherapy.[33] When chlorpromazine began to be used in treatment facilities such as the VA neuropsychiatric hospital system, treatment practices overall changed rapidly. There is an inverse correlation between the adoption of drugs like chlorpromazine in VA neuropsychiatric facilities and the usage of electroconvulsive and insulin shock therapy in those facilities.[33] While this trend is certainly noteworthy, it cannot be concluded that drugs alone were responsible for it.

However, what can be said with certainty is that the introduction of drugs like chlorpromazine was a hallmark event in the history of schizophrenia. Dr. Douglas Goldman, one of the first to conduct a trial with chlorpromazine for schizophrenia in the United States, said the following at a conference in 1955: “chronic, severe schizophrenic illness, resistant to all other treatments, … has represented a ‘therapeutic no man’s land’ … The application of chlorpromazine in such situations has, however, accomplished results never heretofore achieved.”[33]

At that time, psychiatrists had no concept of the molecular workings of the nervous system and brain, but the clear benefits conferred by drugs no doubt stimulated the curiosity of scientists and psychiatrists alike, ushering in a new paradigm where investigators sought to understand how small molecules could affect behavior. This knowledge could lead to better understanding of the underlying causes of a vast array of mental disorders, thereby serving as a foundation for development of new and effective treatments. In this way, deployment of chlorpromazine and similar drugs for treatment of the mentally ill marks the origin of modern biological psychiatry. When the dopaminergic antipsychotic chlorpromazine was introduced, the neurotransmitter dopamine had not yet been discovered, and it would be over twenty years before the main target receptor of the drug was conclusively elucidated in the mid-1970s (as reviewed by Howes and Kapur[34] as well as by Madras[35]). Today, this target is known as the dopamine D2 receptor.

Characterization of the role of dopamine receptors in antipsychotic effect remains one of the most important events in the development of dopaminergic hypotheses of schizophrenia. In 1975, Seeman and Lee reported a direct relationship between the

clinical effectiveness of antipsychotic drugs and their ability to block dopamine receptors.[36] In keeping with this finding, the idea that schizophrenia resulted from altered dopamine receptor density in general (as reviewed by Howes, McCutcheon, and Stone)[37] became the most prevalent of the time. While many refinements to the understanding of dopamine's involvement in schizophrenia have occurred since then, it is worth noting that all currently approved drugs for schizophrenia are alike in that they share an affinity for the dopamine D2 receptor.[38]

Until recently, the idea that excessive dopaminergic transmission in regions of the forebrain is a major factor underlying schizophrenia has been the basis of prevailing hypotheses concerning the pathophysiology of disorder. However, drugs that target this dopaminergic transmission have only exhibited limited efficacy and carry risks of many severe side effects, as previously mentioned. These issues have driven researchers to identify and characterize dysfunction in additional neurotransmission systems (i.e. glutamate, serotonin) in an effort to develop improved treatments for schizophrenia.[11]

2.2: Glutamatergic Hypotheses: The NMDAR Antagonism Model

In the same way that search for the mechanism of action of known antipsychotic compounds led to the eventual discovery of the dopamine D2 receptor (DRD2) and the dopaminergic hypothesis of schizophrenia, efforts to understand how seemingly pro-psychotic compounds created their effects formed the basis of glutamatergic hypotheses in schizophrenia. Early observations that 1) administration of phencyclidine (PCP) to normal subjects caused schizophrenia-like symptoms belonging to all three

symptom clusters (i.e. positive, negative, and cognitive) and 2) that PCP, when given to chronic schizophrenia patients, seemed to worsen their symptoms as if they had re-entered the acute phase, originally spurred interest in this direction.[39] Other compounds like ketamine have also been found to induce this phenomenon.[40] Both PCP and ketamine are members of the same chemical class (arylcyclohexylamines), and both possess ability to antagonize the N-methyl-D-aspartate receptor (NMDA receptor), an ionotropic glutamate receptor in nerve cells. Notably, it has been demonstrated that the ability of NMDAR blockade to produce symptoms relevant to schizophrenia (e.g. hyperlocomotion in rodents) remains present: 1) in the absence of dopaminergic activity and/or 2) in the presence of dopamine antagonism.[41] In light of these findings, researchers realized that glutamatergic dysfunction in the context of schizophrenia warranted further investigation.

In 1995, Moghaddam and Liu reported on findings that administration of NMDAR antagonists to awake, freely-moving rats increased glutamate efflux in the hippocampus and striatum in a dose-dependent manner.[42] These findings were extended to the prefrontal cortex (PFC) two years later, when further evidence demonstrated that subanesthetic doses of ketamine (i.e. enough to produce cognitive impairment and other symptoms relevant to schizophrenia, but not enough to render the animal unconscious) increase glutamate efflux in the PFC, thereby disturbing normal dopaminergic neurotransmission and creating cognitive deficits.[43] This alteration to normal dopaminergic function in the PFC was partially reversed by the administration of AMPA receptor antagonists, suggesting that AMPA receptors might occupy an intermediary position in the mechanism.[43]

This hypothetical pathway was supported and perhaps further explained by findings that NMDAR antagonists increased the spontaneous activity of cortical neurons. However, this increased activity was marked not by increased firing of already-present coordinated burst potentials, but instead by an increase in randomly distributed single-spike potentials.[44] Authors have described these randomly distributed spikes as "noise" that diminishes the ability of cortical neurons to filter out irrelevant information,[6,45] which ultimately leads to expression of abnormal behavior – as demonstrated in rats whose display of behavioral stereotypy "correlates with increases in random spike activity."[44] While it is not realistic to expect any animal model to mimic all aspects of complex mental disorders such as schizophrenia, in the case of cognitive deficit, the NMDAR antagonism model possesses both face and construct validity.[44] In short, the model's face validity stems from its ability to recapitulate PFC-dependent cognitive impairments similar to those seen in schizophrenia (as detailed by Moghaddam & Jackson[46]); the model's construct validity is afforded by data from postmortem brain[47-49] as well as genome-wide association studies[50] implicating irregularities in NMDAR function among persons with schizophrenia.

2.3: Emergence of mGluR2/3 Agonism as a Treatment Strategy

Based on the idea of NMDAR hypofunction being responsible for cortical glutamatergic hyperfunction as it relates to schizophrenia, a first mode of approach to remedy this dysfunction might be to target NMDARs directly, for example by orthosteric agonists. However, this strategy is not viable because orthosteric agonism of NMDAR

can cause several detrimental effects (e.g. seizures and excitotoxicity).[51] Attempts to target other sites on the NMDAR receptor with positive allosteric modulators (PAMs) have been made, and while these compounds can change NMDAR behavior *ex vivo*, their pharmacokinetics render them ill-suited to perform the same function *in vivo*.[52] These obstacles necessitate the use of other means in order to rescue a more normal glutamatergic tone.

The metabotropic glutamate receptors (mGluRs, Fig. 4) are class C G protein-coupled receptors expressed throughout the central nervous system. They have large, glutamate-binding extracellular domains called “Venus flytrap domains” that are linked to seven transmembrane domains via cysteine-rich domains. Upon ligand binding, their structure allows them to transduce a signal across a neuronal cell membrane to mediate

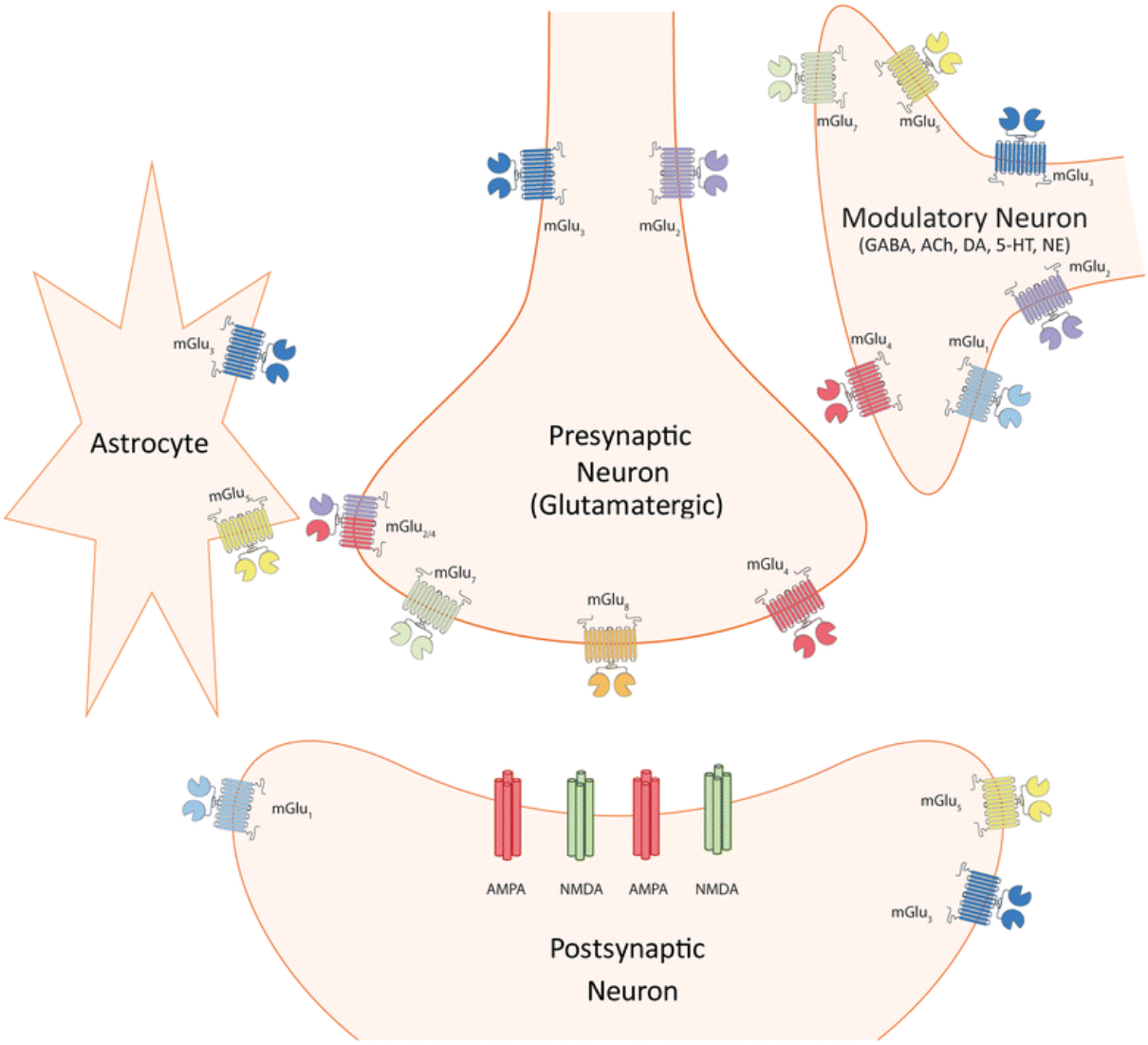


Figure 4. Localization of mGluRs in a synapse. Common locations of mGluRs at a synapse are represented. mGluRs contain characteristic "venus flytrap domains" on their N-terminus that bind glutamate, their endogenous ligand. mGluR2 (purple) is found presynaptically, and can exist as a heterodimer with mGluR4 (red) or as a homodimer. mGluR3 (dark blue) is found both pre- and postsynaptically on glutamatergic neurons, on glia (astrocytes), and on modulatory neurons. Image source: Maksymetz et al., 2017; use permitted under the Creative Commons Attribution License CC BY 4.0.)

intracellular processes. As glutamate is the main excitatory neurotransmitter in the CNS, the presence of mGluRs allows fine-tuning of synaptic behavior and cell excitability by way of second messenger signaling pathways.[25] The mGluRs are divided into three groups on the basis of their amino acid sequences and the signaling pathways that they

activate.[49] Group II is comprised of mGluR2 and mGluR3. These receptors are coupled to $G\alpha_{i/o}$ G protein α subunits, and as such, their signaling pathway leads to inhibition of adenylate cyclase, activation of potassium channels, and inhibition of voltage-sensitive calcium channels.[26]

The group II mGluRs are situated around a synapse in a particular fashion (Fig. 4). Both mGluR2 and mGluR3 are found presynaptically – though not in close proximity to the active zone – and can be activated either by excess glutamate in the synapse or by glutamate released from nearby astrocytes.[53] In addition to presynaptic neurons, mGluR2 and mGluR3 are also found postsynaptically, where mGluR3 in particular can cause hyperpolarization.[54,55] In addition to these locales, mGluR3 is also found on astrocytes, where it has been shown to play a critical role in neuroprotective function.[56] Overall, both receptors are important for inhibition of neurotransmitter release from presynaptic neurons as well as for regulation of synaptic plasticity.[25]

The first indications that group II mGluRs could be targeted as a means to rectify cortical glutamatergic hyperfunction came in 1997, when scientists at Eli Lilly & Co. reported that the activation of group II mGluRs (via a novel exogenous agonist that they synthesized) could normalize excessive glutamate release.[57-58] This compound was LY354740 (Fig. 5a), a potent and highly selective orthosteric agonist for mGluR2/3. LY354740 was quickly followed up by LY379268 (Fig. 5b), a closely-related compound with improved pharmacological and pharmacokinetic properties/characteristics.[59] When subjected to the NMDAR antagonism model, these mGluR2/3 agonists were able to reduce and/or reverse many of the effects created by NMDAR blockade, including

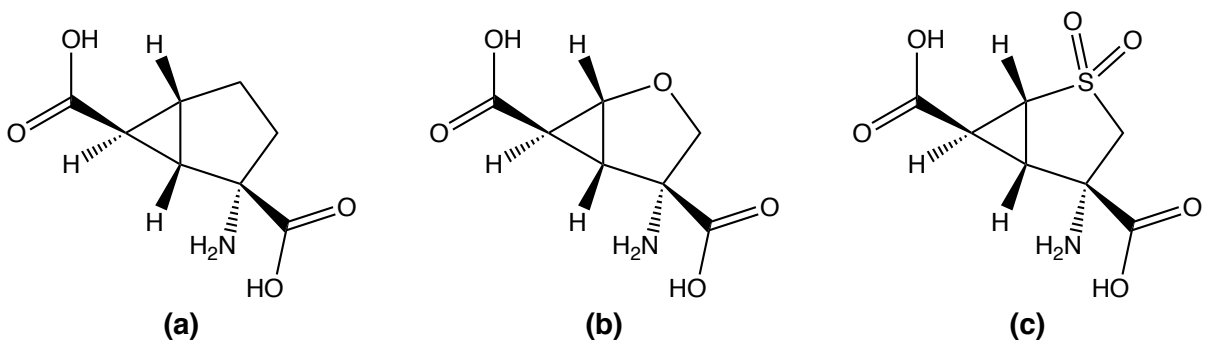


Figure 5. Chemical structures of mGluR2/3 agonists. Structures of three potent and highly selective mGluR2/3 agonists, LY354740 (a), LY379268 (b), and LY404039 (c), are shown.

hyperlocomotion, stereotypies, deficits in working memory, increased cortical glutamate efflux, and increased firing of PFC neurons in rats.[51]

These beneficial effects could be blocked by the mGluR2/3 antagonist LY341495, suggesting that the observed reduction/reversal of such effects was mediated by the mGluR2/3 receptors.[60]

Although the prospects of LY354740 and LY379268 were promising, their development was impeded by pharmacokinetic obstacles, including low oral bioavailability, poor gastrointestinal tract absorption, and insufficient CNS penetrance.[13] The ensuing synthesis of LY404039 (Fig. 5c) represented considerable improvement in these areas. In rodents, bioavailability increased from 10% (LY354740) to 63% (LY404039).[61] The compound maintained the qualities of its predecessors in terms of potency, selectivity, and putative antipsychotic effect. Moreover, it appeared to work through a mechanism distinct from that of the typical and atypical antipsychotics. Perhaps most encouragingly, LY404039 1) did not create any motor side effects in rodents at very high doses and 2) showed potential for efficacy against negative symptoms (i.e. conferring benefits for affective regulation)[13] – traits which are both in stark contrast to currently approved antipsychotic drugs. In humans, oral bioavailability

of LY404039 was lost to a large degree, so Lilly ultimately created the prodrug LY2140023 (pomaglumetad methionil), which has the rather convenient property of being a substrate for a peptide transporter in the human gut. With this strategy, the prodrug is absorbed and then hydrolyzed, ultimately resulting in sufficient concentration of the active parent compound LY404039 in the systemic circulation.[62]

2.4: The mGluR2/3 Agonist Pomaglumetad Methionil in Clinical Trials

On the basis of abundant preclinical evidence for antipsychotic effect, Lilly advanced LY2140023 to human clinical trials. After establishing safety and tolerability, the first phase II study[14] was initiated. The primary objective of this multicenter, randomized, and double-blind trial was to assess whether LY2140023 was superior to placebo in treating patients with schizophrenia as measured by the Positive and Negative Syndrome Scale (PANSS – as developed by Kay, Fiszbein, and Opler[63]) over a 28-day period. The atypical antipsychotic olanzapine was included as an active control. 118 patients completed the 28-day treatment period, with encouraging results:

> patients treated with LY2140023 showed statistically significant improvements in both positive and negative symptoms of schizophrenia compared to placebo, suggesting that LY2140023 might be considered a broad spectrum antipsychotic. Treatment with LY2140023, as well as with olanzapine, was generally safe and well-tolerated. Notably, LY2140023 treatment did not appear to be associated with the common dopamine D2-related adverse events of extrapyramidal motor adverse events, hyperprolactinemia, nor was it associated with weight gain.[62]

In light of these positive results, further clinical trials were warranted and began to be organized.

Next, a larger (*n* = 669) phase II study[15] aimed at assessing efficacy over a larger dose range of LY2140023 was conducted. Like the first trial, this multicenter, double-blind, and randomized trial measured primary outcome with the PANSS, used olanzapine as an active control, and lasted 28 days. Results from this trial were considered inconclusive when neither olanzapine nor any dose of LY2140023 separated from placebo. Confusingly, the placebo group in this study demonstrated significant improvement as measured by PANSS, whereas the placebo group in the earlier study showed no significant change.[62]

The next trial sought to establish long-term safety, tolerability, and efficacy of LY2140023. To do so, this study[16] lasted 24 weeks and compared LY2140023 to the current "standard of care" (SOC) drugs olanzapine, risperidone, and aripiprazole. To assess tolerability, "discontinuation due to adverse events" was used as the primary outcome measure. Antipsychotic efficacy was assessed by the PANSS scale – though it is important to note that a placebo arm was not included. While LY2140023 did not create motor side effects (as the SOC drugs did), it failed to achieve any long-term efficacy benefit over SOC drugs (as measured by PANSS), which outperformed LY2140023 in this regard.[62]

In 2013, a smaller phase Ib study[17] was published. This trial had a different focus: whether LY2140023 could confer negative symptom reduction when added on top of ongoing therapy with atypical antipsychotics. The results were yet another blow to the prospects of LY2140023, as it failed to achieve any benefit over placebo in this paradigm.

In 2014, results from the largest trial ever conducted with LY2140023 were published.[18] This phase II/III study randomized patients (*n* = 1013) to one of four categories: placebo, LY2140023 (2 different doses), or risperidone. The primary outcome was measured by improvement in PANSS scores after six weeks. Unfortunately, neither dose of LY2140023 separated from placebo, while risperidone did separate. In their report on the results of the study, the authors concluded: "further understanding of the role of glutamate as a therapeutic target in schizophrenia is needed."[18] After these repeated failures, Eli Lilly & Co. terminated the development of LY2140023.

2.5: Newer Insights on mGluR2 in the Context of Schizophrenia

Over the last ten years, numerous discoveries concerning oligomeric structure, behavior, and regulation of mGluR2 have been made. Many of these findings have immense functional relevance not only for the receptor itself, but also to the glutamate hypothesis and to schizophrenia in general. Furthermore, these recent advances may offer a possible explanation as to why the strategy of mGluR2/3 agonism (in the form of LY2140023) failed to succeed in human clinical trials.

Due to the lack of specific molecular insight on the causes of schizophrenia, researchers have largely relied on drugs to help guide their work. In other words, observations that certain drugs exhibit antipsychotic activity helped investigators focus on their mechanisms of action as a way to understand dysfunctional neurotransmission in the schizophrenia-afflicted brain (e.g. chlorpromazine and DRD2). Likewise,

observations that other drugs were seemingly pro-psychotic (or, psychotomimetic) also guided work in this area (e.g. PCP and NMDAR hypofunction leading to glutamatergic dysfunction in the frontal cortex). Thus, understanding how drugs alleviate or create one or more symptoms of schizophrenia is the primary way that researchers have generated hypotheses concerning dysfunction of major (i.e. dopamine, glutamate) neurotransmission systems.

In keeping with this conceptual framework, clues from other drugs helped further resolve the molecular interactions relevant to the mGluR2/3 agonism treatment strategy. In addition to their action at DRD2, atypical antipsychotics (e.g. clozapine, risperidone) also act as antagonists of the serotonin receptor 2A (5-HT_{2A}R or more simply 2AR). Of further relevance is the knowledge that hallucinogenic drugs such as LSD, DOI, and psilocybin are agonists of the 2AR and cause changes in sensory processing and perception – effects which are blocked by atypical antipsychotics. So, while agonism of mGluR2/3 seems to confer the desired effect, antagonism of other receptors like 2AR achieves this as well. Though different in their targets and pharmacology, both the atypical and glutamate antipsychotics are alike in their end result: antipsychotic action. The juxtaposition of opposing pharmacological action (i.e. agonism vs antagonism) with similar therapeutic result (i.e. antipsychotic effect) begged the question: are the 2AR and mGluR2/3 related in this context? In other words, in the pathway from receptor binding to eventual antipsychotic action, is there a point where their mechanisms converge?

In 2008, González-Maeso *et al.*[28] demonstrated that the 2AR and mGluR2/3 directly interact with one another in the form of a heterodimer. Like mGluR2/3, 2AR is

also a GPCR, however it is a member of a different class and has downstream signaling cascades distinct from those of mGluR2/3. Importantly, the interaction between the two receptors was shown to have functional consequences. The first suggestion of functional interplay came from findings in mice. First, mice with a global 2AR knockout (*htr2A*$^{-/-}$) were found to express mGluR2 to a much lower extent than wildtype mice. Rescue of 2AR expression reversed this phenotype. Second, the mGluR2/3 agonist LY379268 increased the affinity of LSD and DOI for their 2AR binding site. Conversely, the 2AR agonist DOI decreased the affinity of LY379268 for its binding site on mGluR2/3. Third, the presence of specific downstream signaling markers of 2AR activation by hallucinogens was abolished by LY379268. Lastly, when treated chronically with atypical antipsychotics, expression levels (mRNA and receptor density) of 2AR and mGluR2 in mouse frontal cortex fell significantly. Interestingly, in the 2AR knockouts, chronic treatment with atypical antipsychotics did not alter the mGluR2 level, indicating that the 5-HT_{2A} receptor was necessary for the downregulation of mGluR2 by this treatment.[28] These findings were congruent with studies showing different levels of 2AR and mGluR2 in postmortem human brains from untreated and antipsychotic-treated schizophrenia patients,[28] and thus further reiterated the functional implications of the heterodimeric complex in the context of schizophrenia and its treatment.

Though the 2AR was known to be required for the downregulatory effect of chronic clozapine treatment on mGluR2, the specific mechanism through which this proceeded was unknown until 2012, when histone deacetylase 2 (HDAC2) was convincingly demonstrated to be the main effector in this process. Kurita *et al*.[20] showed that chronic treatment with clozapine causes a 2AR-dependent upregulation of HDAC2,

leading to increased HDAC2 binding to the promoter of the *mGlu2* gene, ultimately resulting in decreased expression of mGluR2 in both mouse and human frontal cortex. Concordantly, adjunctive treatment with the broad-spectrum HDAC inhibitor suberanilohydroxamic acid (SAHA) nullified this phenomenon in mice,[20] in keeping with clinical observations that HDAC inhibitors conferred additional antipsychotic benefit when given in conjunction with atypical antipsychotics.[64-66]

More recently, HDAC2 has also been implicated as a driver of a number of neurological side effects caused by chronic treatment with atypical antipsychotics, such as decreases in mature synapse number.[22] Along the same lines, since mGluR2 is crucially important for the induction of long-term depression in the cortex as well as at synapses between mossy fibers and hippocampal CA3, its dysregulation contributes to impairments in synaptic plasticity.[67] Thus, it is conceivable that the therapeutic benefits of chronic treatment with atypical antipsychotics may be restrained by the negative effects of the HDAC2 upregulation that these drugs induce.

Finally, the downregulation of mGluR2 by atypical antipsychotics has relevance to the interpretation of the performance of LY2140023 in clinical trials. Citing the previously discussed 2012 work by Kurita *et al.*, investigators at Eli Lilly gave due consideration to this possibility in a post-hoc analysis of the trials, writing:

> The possible epigenetic influence of prior atypical anti-psychotic treatment to down-regulate the mGlu2 receptor is a phenomenon that has only recently been demonstrated in the laboratory and has not yet been accommodated into preclinical models that screen for potential new antipsychotic medications. Thus, the well-characterized ability of pomaglumetad to block the psychosis-like effects of N-methyl-D-aspartate antagonists, such as phencyclidine and ketamine in animals, may not have translated to the clinic as most patients in schizophrenia trials have in fact had substantial previous treatment exposure to atypical antipsychotic drugs or, more specifically, to drugs with prominent 5-HT_{2A}

> antagonist attributes … It is an intriguing possibility that the intimate relationship between 5-HT_{2A} and mGlu2 receptors extend to epigenetic interactions and that chronic 5-HT_{2A} receptor blockade might lead to a down-regulation of mGlu2 receptor levels. Thus, even if a hyper-glutamatergic state contributes to schizophrenic symptoms, a treatment targeted to mGlu2 receptor activation would yield reduced efficacy if the receptor target levels were notably reduced. Consistent with this hypothesis, our post hoc analyses indicated that patients with chronic exposure to agents containing potent 5-HT_{2A} receptor antagonism exhibited a lack of therapeutic response to pomaglumetad, which suggests that preservation of the mGlu2 receptor target is necessary to effect an antipsychotic response to pomaglumetad.[23]

Accordingly, it is possible that patient selection – conducted without knowledge and/or consideration of the effects of chronic atypical antipsychotics on mGluR2 – could be responsible for at least some of the failure of LY2140023 to succeed in clinical trials. As such, design of future clinical studies with glutamatergic antipsychotics should account for recent elucidation of the role of HDAC2 in downregulation of mGluR2 following chronic administration of atypical antipsychotics.

Chapter 3: Methods and Development

3.1: Generation of HDAC2 Conditional Knock-out Mice

In mice, HDAC2 is expressed ubiquitously[68], and global deletion of widely-expressed *Hdac2* results in either embryonic lethality or perinatal lethality due to impaired development and a variety of defects.[22] Therefore, another approach is necessary to delete HDAC2 expression in cortical pyramidal neurons. *CaMKIIα* is a marker of forebrain glutamatergic pyramidal neurons.[69] Expression of this marker in these neurons begins 10-14 days after birth.[70] Therefore, when deployed as part of a Cre-Lox recombinase system, *CaMKIIα* can confer both temporal and cell type specificity. To take advantage of this, transgenic C57BL/6 mice heterozygous for *CaMKIIα-Cre* were bred with mice homozygous for $Hdac2^{loxP/loxP}$. Consequently, offspring that express *Cre* will delete *Hdac2* in neurons expressing *CaMKIIα*. Conversely, offspring that do not express *Cre* will have normal function of HDAC2. This approach bypasses developmental impairments and abnormalities associated with global *Hdac2* deletion and generates a viable mouse line with conditional knockout of HDAC2 (HDAC2-cKO). Offspring were born at near-expected Mendelian ratios.

3.2: Mouse Genotyping

At weaning (28 days after birth), ear punches were obtained from each mouse and frozen at -80°C. To isolate DNA, the tissue was placed in a PCR tube containing 200µL GNT-K buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 0.01% gelatin, 0.45% Igepal® GA-630 [Sigma I3021], 0.45% Tween® 20 [Sigma P1379] and 100 µg/ml proteinase K), which was incubated at 55°C for 2 hours and then heated at 95°C for 15 minutes. For PCR amplification, 22 µL of primer mixture containing primers for *CaMKII-Cre* (Table 1) or *Hdac2*$^{loxP/loxP}$ (Table 2) each at a concentration of 100 µM was added to a PCR tube containing a PuReTaq Ready-To-Go PCR Bead (Amersham 27-9557-01), followed by the addition of 3 µL of isolated DNA. A representative photograph of DNA electrophoresis gels corresponding to genotyping results for *CaMKII-Cre* and *Hdac2*$^{loxP/loxP}$ can be found in Figures 6 and 7, respectively.

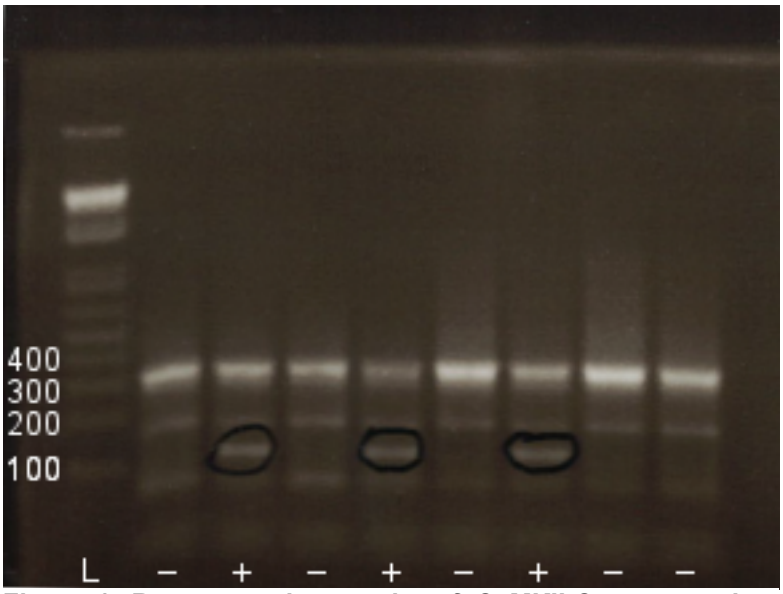


Figure 6. Representative results of *CaMKII-Cre* genotyping. Photograph of agarose gel indicating if young mice were positive (+, black circles) or negative (-) for the *Cre* transgene (~100 bp). The band at ~324 bp corresponds to the internal positive control.

Table 1. PCR Primers and Protocol Used for *CaMKII-Cre* Genotyping.

PCR Primers

Primer	Primer Type	Sequence (5' → 3')
oIMR1084	Transgene	GCG GTC TGG CAG TAA AAA CTA TC
oIMR1085	Transgene	GTG AAA CAG CAT TGC TGT CAC TT
oIMR7338	Internal Positive Control	CTA GGC CAC AGA ATT GAA AGA TCT
oIMR7339	Internal Positive Control	GTA GGT GGA AAT TCT AGC ATC ATC C

PCR Protocol

Step	Temp. (°C)	Time	Note
1	94	3 min	
2	94	30 sec	
3	51.7	1 min	
4	72	1 min	Repeat steps 2-4 35 times
5	72	2 min	
6	10	∞	Final hold

Table 2. PCR Primers and Protocol Used for *Hdac2*$^{loxP/loxP}$ Genotyping.

PCR Primers

Primer	Primer Type	Sequence (5' → 3')
HDAC2 WT FOR	Reaction 1: WT allele	GCA CAG GCT ACT ACT GTG TAG TCC
HDAC2 REV	Reaction 1: WT allele	CCA CCA CTG ACA TGT ACC CAA C
HDAC2 MUT FOR	Reaction 2: *loxP* allele	GTC CCT CGA CCT GCA GGA ATT C
HDAC2 REV	Reaction 2: *loxP* allele	CCA CCA CTG ACA TGT ACC CAA C

PCR Protocol

Step	Temp. (°C)	Time	Note
1	94	2 min	
2	94	15 sec	
3	60	30 sec	
4	72	40 sec	Repeat steps 2-4 30 times

5	72	5 min	
6	10	∞	Final hold

3.3: Chronic Treatment of Mice

Adult male and female C57BL/6 mice (10-20 weeks old) were chronically treated with daily intraperitoneal injection of clozapine (10 mg/kg – obtained from Tocris Cookson Inc.) or vehicle for 21 consecutive days. Clozapine was dissolved in DMSO supplemented with a minimal amount of acetic acid and suspended in saline. 24 hours after the final treatment was administered, mice were sacrificed by cervical dislocation immediately followed by decapitation. Brains were removed, washed briefly in phosphate-buffered saline (pH 7.4), and dissected for bilateral frontal cortex (bregma 1.90 to 1.40 mm) on an ice-cooled petri dish lid. Tissue was promptly frozen in a 1.5 mL microcentrifuge tube at -80C. Animals were housed at 12 h light/dark cycle at 23°C with food and water ad libitum. Experiments were conducted in accord with NIH guidelines, and were approved by the Virginia

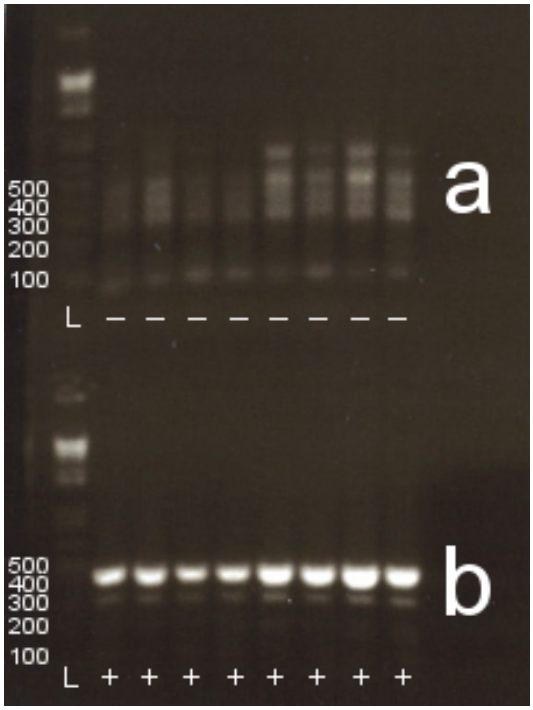


Figure 7. Representative results of Hdac2$^{loxP/loxP}$ genotyping. Photograph of agarose gel indicating if young mice were positive or negative for wild-type *Hdac2* (a) and mutant *Hdac2$^{loxp/loxp}$* (b), both ~450 bp. All mice used in the study efficiently expressed *Hdac2$^{loxp/loxp}$*. Mice that expressed the wild-type *Hdac2* (not shown) were excluded from the study.

Commonwealth University Animal Care and Use Committee. All efforts were made to minimize animal suffering.

3.4: Preparation of Membranes

Dissected brain tissue was frozen at -80°C for a minimum of 24 hours. After thawing on ice, tissue was sheared with a syringe (23G needle) in assay buffer (pH 7.7, containing, in mM: 20 HEPES, 10 $MgCl_2$, 2 EGTA, 100 NaCl) and further homogenized in a 5 mL Teflon-glass grinder. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C. Next, the supernatant was centrifuged at 40,000 x g for 20 min at 4°C. The resulting pellet was washed with fresh assay buffer and centrifuged twice more at 40,000 x g for 20 min at 4°C before storage at -80°C. Preparation of membranes from HEK293 cells stably expressing mGluR3 was performed in the same manner.

3.5: Development of Agonist-stimulated [^{35}S]GTPγS Binding Assay

Early attempts to perform LY379268-stimulated [^{35}S]GTPγS binding assays in native tissue membrane preparations were not successful. Puzzlingly, glutamate-stimulated [^{35}S]GTPγS binding was working as expected, indicating that the issue most likely had something to do with LY379268 and/or the mGluR2 receptor. Early attempts at this assay used native tissue membranes prepared according to previous literature.[71] After quantification by the Bradford method, 10 μg of protein (i.e. membranes) was added to individual wells on a 96-well plate with the following reaction conditions: assay buffer (pH 7.4) containing 20 mM HEPES, 3 mM $MgCl_2$, 100 NaCl, 5 μM GDP, 0.05 nM

[^{35}S]GTPγS (Perkin-Elmer), and 20 µL of either cold GTPγS (final concentration 10 µM – for assessment of non-specific binding), vehicle (for assessment of basal binding), or LY379268 (Tocris-Cookson Inc.) at varying concentrations for a total reaction volume of 200 µL. Components were added to wells individually in the following order: 1) GDP; 2) assay buffer; 3) either cold GTPγS, vehicle, or LY379268; 4) [^{35}S]GTPγS; and 5) membranes. After incubation for 1 hour at 30°C, the reaction was stopped by filtration (FilterMate Harvester, Perkin-Elmer) with a glass fiber filter (Printed Filtermat A, Perkin-Elmer) and washed 6 times with ice-cold assay buffer. The glass filter was dried at 55°C for 1h, soaked in scintillation liquid (Betaplate Scint, Perkin-Elmer), and the radioactivity counted by a Microbeta2 counter (Perkin-Elmer). In troubleshooting the many failures with LY379268, a different mGluR2/3 agonist, LY354740, was used. With either agonist, the best results able to be obtained with these conditions were unsatisfactory (Fig. 8) and not sufficiently robust for analysis of chronically treated mice.

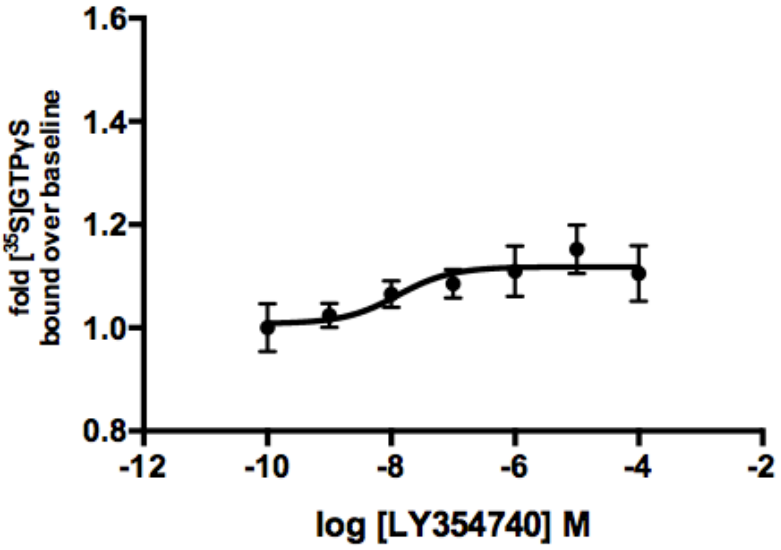


Figure 8. Early attempt at LY354740-stimulated [^{35}S]GTPγS binding in mouse frontal cortex. A weak signal amounting to only 11.8% over basal was the best I was able to obtain throughout all of my attempts with the older protocol. Experiment was performed in triplicate. Error bars are ± SEM. Nonlinear regression was performed in GraphPad Prism (ver. 6).

After studying the [^{35}S]GTPγS assay in detail[72] and locating an example[73] using conditions slightly different from those most commonly encountered in the literature, I

attempted to perform the assay in a new way. In addition to changing the reaction conditions (Table 3), I also developed a “master-mix”-driven approach, where as many components as possible are mixed prior to plating, as opposed to adding all the components to each well separately. This drastically reduces variability across wells/replicates as well as limiting opportunities for mistakes. The first step is

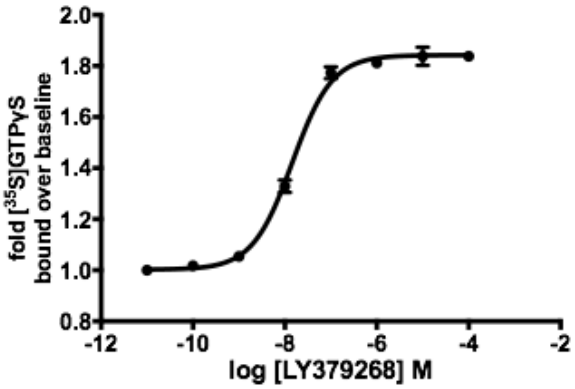
Table 3. Summary of improvements to protocol for agonist-stimulated [^{35}S]GTPγS binding assay for mGluR2 in mouse frontal cortex.

Reaction Component	Previous Condition	Change	Improved Condition
pH	7.4	▲	7.7
HEPES	20 mM		20 mM
NaCl	100 mM		100 mM
$MgCl_2$	3 mM	▲	10 mM
EGTA	none	+	2 mM
GDP	5 µM	▲	30 µM
[^{35}S]GTPyS	0.05 nM	▲	0.3 nM
GDP:[^{35}S]GTPyS	100,000 : 1		100,000 : 1
Protein/well	10 µg		5-10 µg
Incubation	1 hr, 30°C	▼	45 min, RT

to load the plate with 20 µL of either 1) cold GTPγS (for quantification of non-specific binding, final concentration 10 µM); 2) vehicle (i.e. whatever substance the agonist or test compound is dissolved in); or 3) varying concentrations of test compound. Next, calculations were performed in order to create a master mix (20 mM HEPES, 10 mM $MgCl_2$, 2 mM EGTA, 100 mM NaCl, 30 µM GDP, 0.3 nM [^{35}S]GTPγS) at 90% of the total volume required. The remaining 10% of the volume was contributed by the addition of

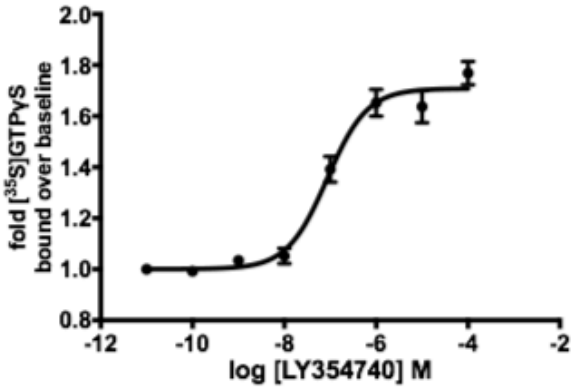
membranes suspended in assay buffer at the desired concentration. Once membranes are added, the mixture is plated immediately: 180 µL is added to each well to create a reaction volume of 200 µL. As before, the reaction was stopped by harvesting the plate through a glass fiber filter with 6 washes of ice-cold assay buffer, and the radioactivity was counted. This new approach yielded very robust results (Fig. 9), and worked well with both LY379268 (Fig. 9a) and LY354740 (Fig. 9b). This approach was used for all assays thereafter.

(a)



log(agonist) vs. response (three parameters)	
Best-fit values	
Bottom	1.002
Top	1.842
LogEC50	-7.838
EC50	1.452e-008
Span	0.8402
Std. Error	
Bottom	0.01347
Top	0.01192
LogEC50	0.04960
Span	0.01736

(b)



log(agonist) vs. response (three parameters)	
Best-fit values	
Bottom	1.000
Top	1.708
LogEC50	-7.069
EC50	8.538e-008
Span	0.7076
Std. Error	
Bottom	0.02253
Top	0.02621
LogEC50	0.1115
Span	0.03342

Figure 9. Improved [^{35}S]GTPγS assay conditions create robust concentration-response curves. Results from agonist-stimulated [^{35}S]GTPγS binding assays performed with the mGluR2/3 agonists LY379268 (a) and LY354740 (b) in membranes from mouse frontal cortex are shown. Parameters resulting from nonlinear fit are shown beside each curve. Both experiments were performed in triplicate. Error bars are ± SEM. Nonlinear regression was performed in GraphPad Prism (ver. 6) as described in section 3.6.

I have since formalized a protocol for the agonist-stimulated [^{35}S]GTPγS binding assay in native tissue, and have distributed it to members of my laboratory, who have used it with success.

3.6: Data and Statistical Analysis

Agonist-stimulated [^{35}S]GTPγS binding data was analyzed by nonlinear regression performed by curve fitting software (GraphPad Prism, version 6) using the parameters listed in Table 4.

Table 4. Curve fitting parameters used throughout this study.

Equation	log (agonist) vs. response (three parameters)
Model	$Y = Bottom + (Top - Bottom) / (1+10^{logEC50-X})$
Constraints	Hill slope constrained to 1

Fold changes were normalized to [^{35}S]GTPγS binding in the absence of the mGluR2/3 agonist. An extra-sum-of-squares (F-test) was used to determine statistical difference for simultaneous analysis of binding curves (Fig. 13). Differences between experimental conditions were assessed by two-way ANOVA followed by Bonferroni's post hoc test (Table 5). Area under curve analysis (Fig. 14) was conducted in GraphPad Prism (ver. 6) on each individual curve for the chronic treatment experiment (Fig. 13). Total area was used, and baseline was set to y = 1.0.

Chapter 4: Results

4.1: Validation of [^{35}S]GTPγS Assay

In addition to reproducibility, validation of the LY379268-stimulated [^{35}S]GTPγS binding assay required demonstration of the following: 1) [^{35}S]GTPγS binding is concentration-dependent as well as saturable with respect to agonist (Figs. 9a, 11, 12);

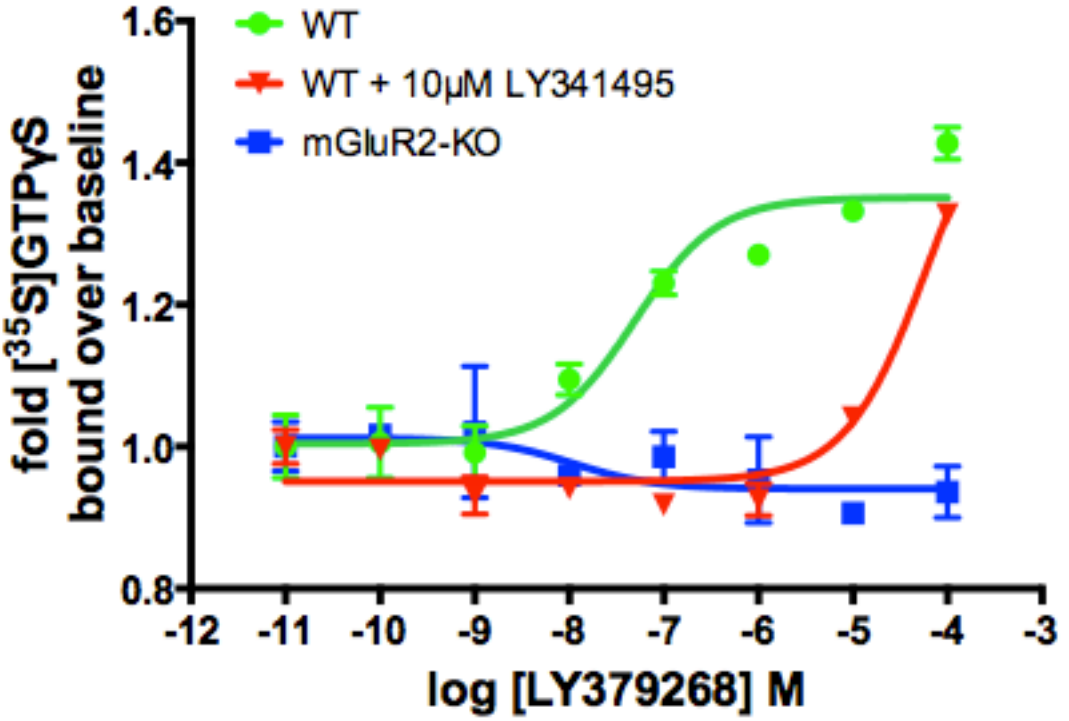


Figure 10. LY379268-stimulated [^{35}S]GTPγS binding activity is blocked by the mGluR2/3 antagonist LY341495 and is lost in mGluR2-KO mice. In wild type mice, LY379268 (green circles) displayed concentration-dependent [^{35}S]GTPγS binding, whereas LY379268 in the presence of 10 μM LY341495 (red triangles) did not. In mGluR2-KO mice, concentration-dependent [^{35}S]GTPγS binding in response to LY379268 was not observed (blue squares). All assays were performed in triplicate with membrane homogenate derived from either wild type or mGluR2-KO mouse frontal cortex. Error bars are ± SEM. Nonlinear regression was performed in GraphPad Prism (ver. 6) as detailed in section 3.6.

2) the agonist must be blocked by the appropriate antagonist (LY341495, Fig. 10). Both of these requirements were met.

4.2: Specificity Between mGluR2 and mGluR3

Specific pharmacological study of either mGluR2 or mGluR3 is complicated by the fact that their respective binding sites for glutamate are extremely similar – there are no currently available orthosteric agonists that exhibit sufficient selectivity between mGluR2 and mGluR3. As such, commercially available orthosteric agonists are commonly referred to as "mGluR2/3 agonists." While stable/transfected cell lines can be used to express one of the two receptors and study it in isolation, investigators wishing to parse apart mGluR2- or mGluR3-specific effects in an intact physiological system most likely must rely on knockout animals.

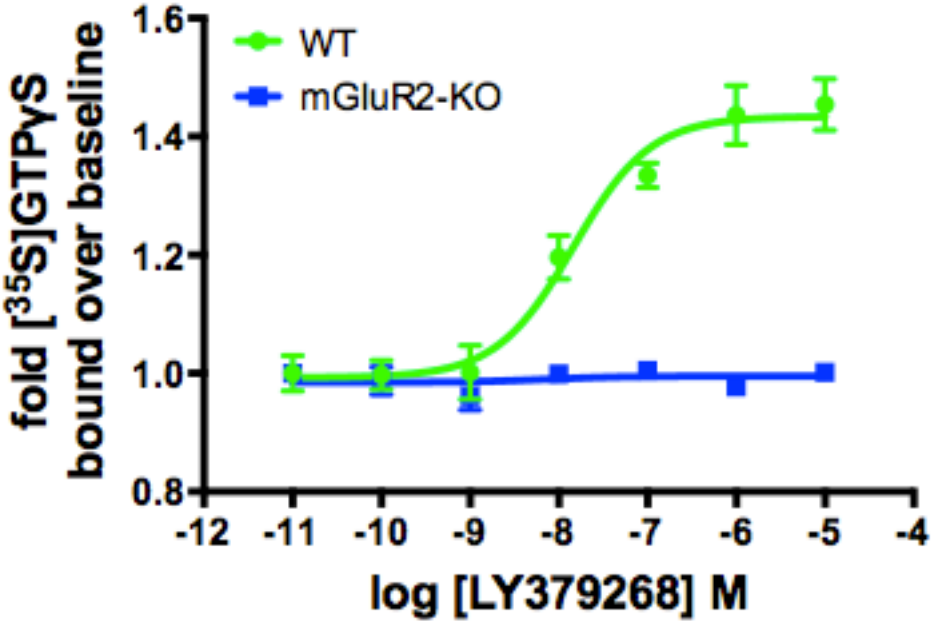


Figure 11. mGluR3 does not display functional activation in the LY379268-stimulated [^{35}S]GTPγS binding assay. Membranes from wild type mouse frontal cortex (green circles) displayed concentration-dependent [^{35}S]GTPγS binding, but membranes from mGluR2-KO mice, which still express mGluR3, did not generate any signal under the same conditions. Both assays were performed in triplicate. Error bars are ± SEM. Nonlinear regression was performed in GraphPad Prism (ver. 6) as detailed in section 3.6.

In some early experiments, I used mGluR2 knockout mice (mGluR2-KO) in an effort to assess the relative contributions of mGluR2 and mGluR3 in the context of my LY379268-stimulated [^{35}S]GTPγS binding assay (as developed for mouse frontal cortex). On first attempt, there was no activity at all in these mice (Fig. 10), which was surprising for a number of reasons. First, both mGluR2 and mGluR3 are said to be G_i-coupled, which is ideal for the [^{35}S]GTPγS assay. Second, LY379268 possesses nanomolar affinity at both receptors. Third, mGluR3 is thought to be expressed throughout all cortical layers in the forebrain.[74] With this in mind, I tried again with another mGluR2-KO mouse, and obtained very similar results (Fig. 11).

After reading a recent report stating that chloride acts as an agonist at all Group II and III metabotropic glutamate receptors except mGluR2,[75] I wondered if this might be the reason why I was unable to see any signal. My reasoning was that if chloride does, in fact, act as an agonist at mGluR3, then the high amounts of chloride in the assay buffer might already be reaching maximal effect at mGluR3 and addition of further agonist in the form of LY379268 would not produce any further [^{35}S]GTPγS binding. The simple removal of NaCl from the buffer dropped the total chloride anion concentration from 120 mM (100 mM NaCl + 10 mM $MgCl_2$ = 120 mM Cl^-) to 20 mM. This change seemed to marginally improve results, going from no signal to a meager 7% over basal (data not shown). However, the removal of sodium drastically increases basal binding and confounds results, limiting any conclusions that could be drawn from them. Both sodium and magnesium are important in the reaction, so I attempted to use other sources for them that did not contain chloride anion (i.e. sodium gluconate and magnesium $gluconate_2$) while holding other assay conditions constant. Under these

conditions, both LY379268 and glutamate failed to produce any signal in mGluR2-KO frontal cortex (data not shown).

Interestingly, I do not seem to be alone in my issues with this assay at mGluR3 in native membrane preparations. Despite [^{35}S]GTPγS binding being well-characterized for mGluR2 in native tissue membranes, I have been unable to locate any literature reports where this assay is demonstrated in native tissue membrane preparations with mGluR3 alone – although it has been demonstrated in transfected cells. Given the widespread use of this assay, commentary on this phenomenon – one that others have likely encountered – is scarce. In a 2008 study reporting on allosteric modulators for mGluR2 and mGluR3, scientists at Merck addressed this problem:

> Despite finding [multiple allosteric modulators and agonists] to be active … in human mGluR3 recombinant membranes as well as in transiently transfected cells, we did not see biphasic curves with the rat brain membranes. A recent study[74] gives a very detailed report on the distribution of mGluR2 and mGluR3 in the rat forebrain. The two receptors seem to be fairly evenly distributed throughout the forebrain, suggesting that making large-scale membrane preparations from specific brain regions to favor mGluR2 or mGluR3 would be very difficult. However, it would seem from their study that sufficient levels of mGluR3 should have been present in the membranes prepared from rat whole brain to see an agonist or allosteric modulator response. Our recombinant mGluR3 membranes had an EC_{50} value of just >100 nM (data not shown), and it is difficult to remove endogenous glutamate in the rat brain preparations to levels below 1 μM to use for mGluR2 testing. It is likely that residual glutamate was already near the mGluR3 E_{max} value before the addition of glutamate. Therefore, at the 1 μM concentration of glutamate we used in the assay, the native rat brain mGluR3 would likely have been fully activated by residual glutamate. Any additional mGluR3 activity caused by the compounds would be above the 100% glutamate response (i.e., from 100 to 110%) … Attempts to further remove the residual glutamate from the rat brain preparations resulted in increasing loss of activity.[76]

If retention of endogenous glutamate in membrane preparations is, in fact, the source of the issue, I reasoned that I should be able to use the orthosteric mGluR2/3 antagonist

LY341495 in increasing concentration to compete with the retained endogenous glutamate and decrease the level of basal [^{35}S]GTPγS binding. However, the potent antagonist had no effect at concentrations up to and including 1 μM in native membrane preparations from the frontal cortex of mGluR2-KO mice (data not shown).

At this point, it began to seem as though the assay, as performed, was specific for mGluR2. To test this, I performed a LY379268-stimulated [^{35}S]GTPγS binding experiment in wild type mice where half of the replicates also contained ML 337, a

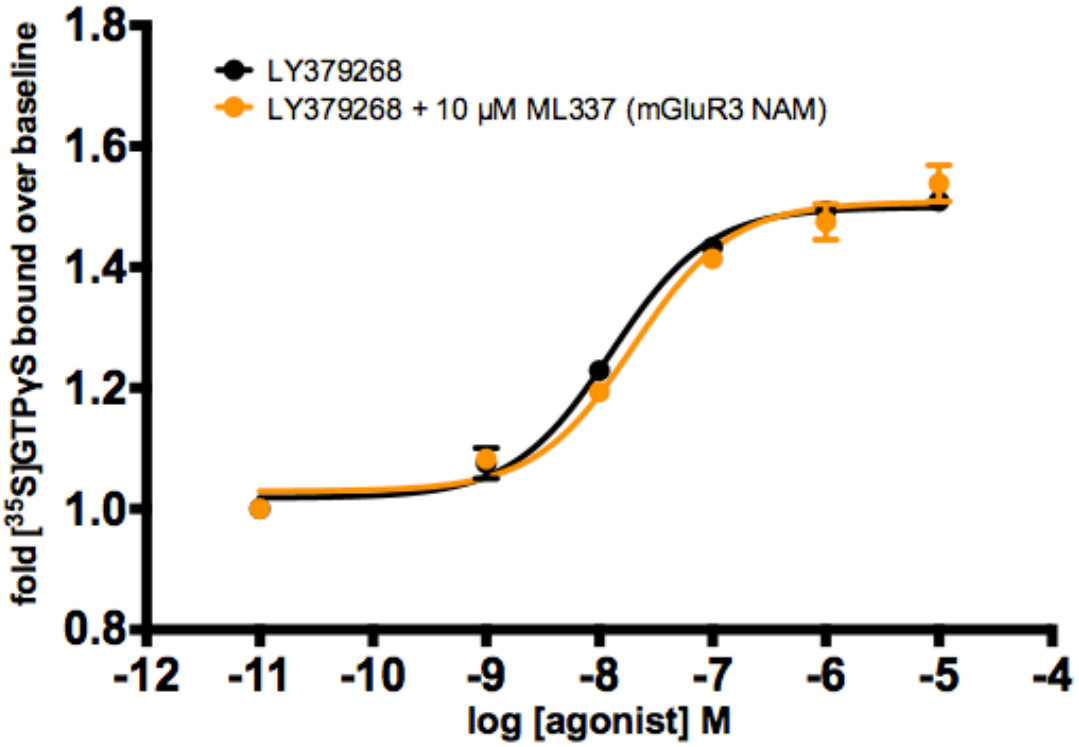


Figure 12. LY379268-stimulated [^{35}S]GTPγS binding in wild type mouse frontal cortex is unaffected by ML 337. There was no significant difference between LY379268-stimulated binding (black) and LY379268-stimulated binding in the presence of the mGluR3-selective NAM ML 337 (orange). Both assays were performed in triplicate. Error bars are ± SEM. Nonlinear regression was performed in GraphPad Prism (ver. 6) as detailed in section 3.6.

selective negative allosteric modulator (NAM) of mGluR3, at a concentration of 10 μM. If mGluR3 was contributing to the overall signal from LY379268 in mice containing

mGluR2 and mGluR3, then the presence of the mGluR3 NAM should decrease [^{35}S]GTPγS binding in some amount dependent on what relative proportion of the overall signal was due to mGluR3. Results of this experiment showed that mGluR3 is not contributing to the overall signal in any significant manner (Fig. 12), lending further support to the idea of an mGluR2-specific assay.

Due to the limited amount of mice available, another system in which to conduct further investigation into the nature of mGluR3 in agonist-stimulated [^{35}S]GTPγS binding was necessary. I shifted my focus to HEK293 cells stably expressing mGluR3 cells in an attempt to see if I could identify conditions that might translate into success in native tissue membranes. The best results that could be obtained were 13% over basal (data not shown), which is still undesirably low. Although I cannot offer an explanation as to why mGluR3 does not generate signal in native tissue membranes, in light of these findings overall it seems that the [^{35}S]GTPγS binding assay, at least as performed in native membranes with the conditions present in this study, is specific to mGluR2 despite the use of the mGluR2/3 agonist LY379268.

4.3: Effect of Chronic Clozapine on mGluR2 G Protein Coupling

Considering the well-established downregulation of mGluR2 following chronic exposure to atypical antipsychotics, I hypothesized that chronic treatment with clozapine, via an HDAC2-mediated mechanism, would reduce functional activation of mGluR2 at the level of its G protein (i.e. reduced G protein coupling). In order to test this, the mGlu2/3 receptor agonist LY379268 was used to perform a series of agonist-

stimulated [^{35}S]GTPγS binding assays in frontal cortex membrane preparations from control and HDAC2-cKO mice. In untreated wild-type mice used to confirm validity of the assay before proceeding with chronic treatment, LY379268 induced binding of the hydrolysis-resistant [^{35}S]GTPγS to the Gα subunit coupled to mGluR2 in a concentration-dependent fashion, and was blocked by the mGluR2/3 antagonist LY341495.

For the chronic treatment mice, the LY379268-stimulated binding assays generated concentration-response data for each mouse, which was analyzed by nonlinear regression to build concentration-response curves and derive pharmacological parameters such as potency (EC_{50}) and efficacy (E_{max}). As expected, control mice chronically treated with clozapine showed concentration-response relationships significantly different from control mice

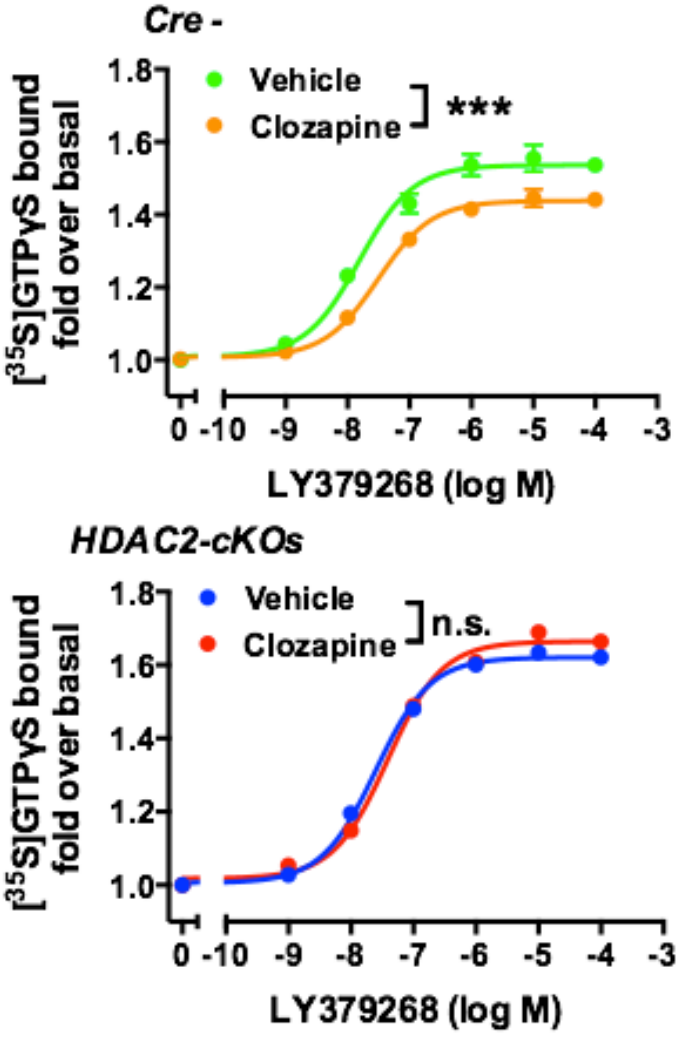


Figure 13. Chronic clozapine decreases LY379268-stimulated [^{35}S]GTPγS binding in the frontal cortex of control mice, an effect that is lost in HDAC2-cKOs. Shown above are concentration-response curves depicting LY379268-stimulated [^{35}S]GTPγS binding in native tissue membrane preparations of frontal cortex from control mice (a) and HDAC2-cKO (b) chronically treated with either clozapine or vehicle (saline). All mice were treated once daily with intraperitoneal injections of either clozapine (10 mg/kg) or vehicle for 21 consecutive days. Mice were sacrificed 24 hours after the final injection (n = 12 mice per experimental condition). Least squares F-test (n.s., not significant; ***P<0.001). See also Fig. 14 and Table 5.

chronically treated with saline (Fig. 13a) (least squares F-test, F(3,154)=18,63, $P < 0.0001$). Specifically, in control mice chronically treated with clozapine, both the efficacy and potency of LY379268 were significantly decreased (Fig. 13a and Table 5). As expected, however, these effects were not observed in the HDAC2-cKO mice (Fig. 13b and Table 5) (E_{max}, two-way ANOVA; genotype effect, F(1,44)=67.41, P<0.0001; treatment effect, F(1,44)=1.85, P>0.05; *post hoc*: controls (clozapine vs. vehicle) P=0.030; HDAC2-cKOs (clozapine vs. vehicle) P>0.05) (EC_{50}, two-way ANOVA; genotype effect, F(1,44)=3.415, P=0.071; treatment effect, F(1,44)=12.24, P=0.001; *post hoc*: controls (clozapine vs. vehicle) P=0.003; HDAC2-cKOs (clozapine vs. vehicle) P>0.05). Area under curve analysis (Fig. 14) provides another way of visualizing the data presented in Figure 13 and Table 5.

Table 5. Pharmacological parameters relative to LY379268-induced [^{35}S]GTPγS binding in frontal cortex membrane preparations from control and HDAC2-cKO mice previously treated with chronic clozapine or vehicle.

	Chronic Vehicle			Chronic Clozapine		
	n	E_{max} (fold-over basal ± SEM)	EC_{50} (logM ± SEM)	n	E_{max} (fold-over basal ± SEM)	EC_{50} (logM ± SEM)
Cre -	12	1.557 ± 0.028	-7.733 ± 0.097	12	1.475 ± 0.022[a]	-7.357 ± 0.083[b]
HDAC2-cKOs	12	1.663 ± 0.014	-7.475 ± 0.039	12	1.691 ± 0.008	-7.353 ± 0.049

[a] There is a significant difference (P< 0.05) in E_{max} between the Cre - mice treated with vehicle and the Cre - mice treated with clozapine. [b] There is a significant difference (P< 0.01) in EC_{50} between the Cre - mice treated with vehicle and the Cre - mice treated with clozapine. A two-way ANOVA with Bonferroni's post hoc test was used for statistical comparison.

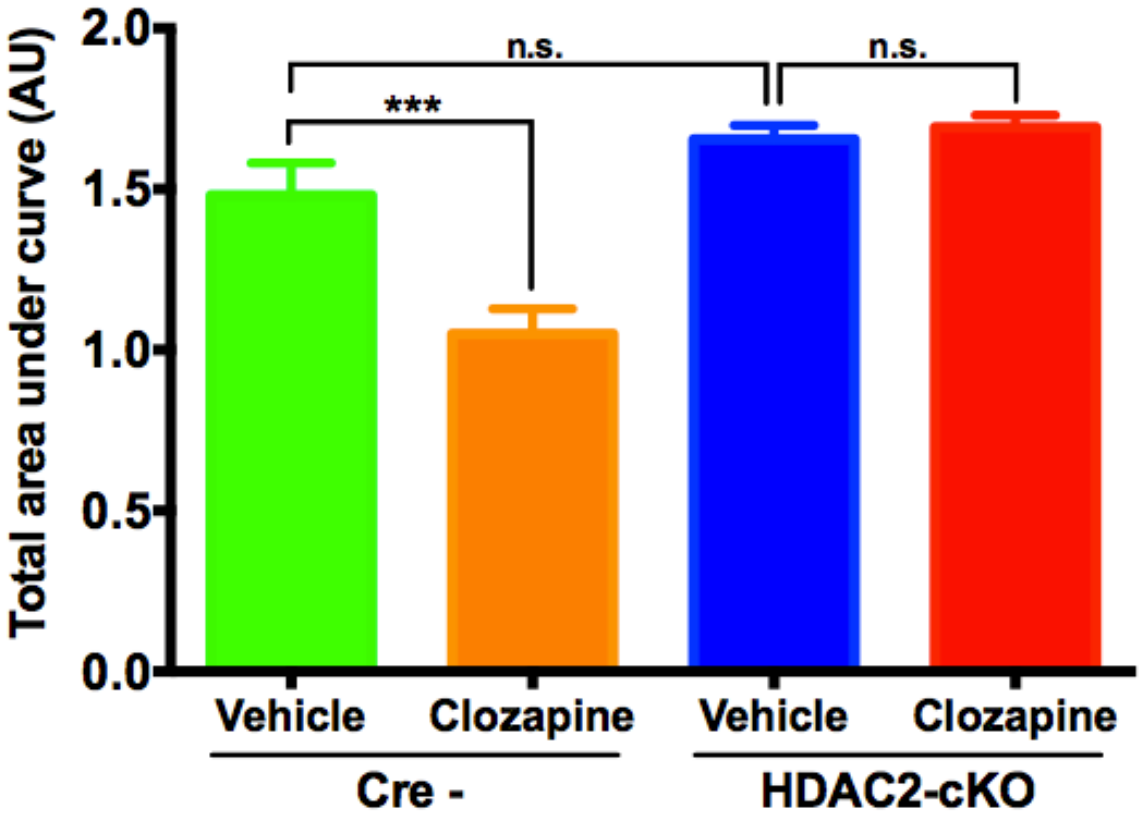


Figure 14. Total area under curve for each experimental condition. Total area under curve was computed for each individual curve in each experimental group (n=12 curves per group) in Prism (ver. 6) as detailed in section 3.6. There was a significant difference in total area under curve between the Cre - mice treated with vehicle and the Cre - mice treated with clozapine which was not observed when comparing the Cre - mice treated with vehicle and the two other groups (two-way ANOVA with Bonferroni's *post hoc* test: ***, $P < 0.001$; n.s., non-significant).

Chapter 5: Discussion

The mechanism by which chronic clozapine treatment upregulates HDAC2 expression and thus causes downstream epigenetic modifications has very recently been further characterized by Ibi *et al*.[22] Decreased signaling from the 5-HT_{2A} receptor diminishes one of the roles of this receptor: activation of the MAPK-ERK signaling pathway. This results in downregulation of *IκBα*, which has the successive effect of increasing nuclear translocation of NF-κB. Inside the nucleus, it binds to the promoter of *Hdac2*, upregulating its expression. The authors state that the repressive changes caused by increased HDAC2 ultimately results in a lower number of mature synapses, decreased synaptic plasticity, and impaired cognitive processing.[22] Consistent with this report, my results support the critical role of HDAC2 in epigenetic changes caused by chronic exposure to atypical antipsychotics. However, my findings are largely situated just beyond where the authors' mechanism ends. In showing that chronic treatment with clozapine decreases G protein coupling of mGluR2, I illuminate a specific functional outcome that has relevance to treatment of schizophrenia.

In neurons, the opposing processes of long-term potentiation and long-term depression (LTD) are critical for the plasticity of synapses. This is especially important in the PFC, where dysfunction (e.g. in mental disorders like schizophrenia) creates deficits in cognition. On a fundamental level, neurons in the cortex rely on dynamic

regulation of the balance between excitation and inhibition in order to function properly. When tight control of this balance is lost, synapses display dysfunctional behaviors.[77] For instance, schizophrenia is marked by improper glutamatergic activity in the PFC, leading to a host of issues including abnormal sensory perception. Given the role of mGluR2 in synaptic LTD, when this receptor behaves aberrantly, there is a diminished ability of neurons to de-potentiate synapses, contributing to a loss of synaptic plasticity. In other words, one of the brain's means of controlling the balance of excitation and inhibition is disrupted. In this way, it is conceivable that loss of normal functional activation of mGluR2 contributes to cognitive impairments that long-term antipsychotic drugs commonly exacerbate.

Throughout the development and optimization of the LY379268-stimulated [^{35}S]GTPγS binding assay, questions surrounding the apparent lack of contribution by mGluR3 to the overall signal produced by an mGluR2/3 agonist began to emerge. While I am unable to offer an explanation as to why this G_i-coupled receptor does not produce robust signals in native tissue membrane preparations, my results (Figs. 10, 11, 12, as well as with LY341495 in mGluR2KO mice) lead me to believe that the [^{35}S]GTPγS assay, at least as performed in native frontal cortex membrane preparations as described here, is specific for mGluR2. Thus, conclusions from the chronic treatment experiment (Fig. 13) apply to mGluR2, but not mGluR3.

It seems that a main target of the newer glutamatergic antipsychotics (mGluR2) is rendered less available and less functional by chronic treatment with current SOC atypical antipsychotics like clozapine. The unfortunate corollary of this phenomenon is that newer drugs being developed (i.e. mGluR2/3 agonists) may not work for those who

are in the most need for new treatments (i.e. patients with treatment-resistant schizophrenia), since clozapine is the only drug approved to treat this population. This has relevance to interpretation of clinical trials, as previously discussed. However, emerging knowledge about how HDAC2 causes these changes is useful in the ongoing quest to develop new and effective treatments. For example, might HDAC inhibition in conjunction with pomaglumetad be a viable strategy for patients who would not otherwise respond? Similarly, could HDAC inhibition in conjunction with clozapine help attenuate the HDAC2 upregulation that, in rodent models, has repeatedly been shown to create cognitive deficits, and may also be responsible for the same effects seen in humans? The drugs that are currently approved to treat schizophrenia have not evolved much in multiple decades despite many problems with efficacy and side effects, presumably owing to the difficulty in identifying viable molecular targets. Perhaps, with the knowledge of the role of HDAC2, we may have identified a new molecular target whose manipulation might represent a new avenue to improve the treatment scenario for the millions of people who suffer from schizophrenia worldwide.

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