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Rachel Reiff April 17, 2012

Regulation of mGluR3 by the Proteasome and Cellular Stress: Implications for the Treatment of Schizophrenia

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Abstract

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Schizophrenia is a serious psychiatric disease that has a profound impact on sufferers, caretakers, and society as a whole. Although many medications that treat symptoms of schizophrenia exist, they have limited efficacy and often produce severe adverse effects. The metabotropic glutamate receptor three (mGluR3) has recently been identified as a potential therapeutic target for the treatment of schizophrenia. Past studies have shown that mGluR3 protein levels may be abnormally low in the brains of schizophrenic patients, and that an agonist for mGluR3 and the related receptor mGluR2 might be useful in alleviating symptoms of schizophrenia. However, much is still unknown about the activity and regulation of mGluR3, and in order to make progress toward developing drugs that target mGluR3, it is first necessary to gain a better understanding of the receptor both in normal states and in disease.

The purpose of this project was to study the ways that mGluR3 is trafficked, degraded, and regulated in a cellular context. Particular focus was given to degradation pathways of mGluR3, as the manipulation of degradation may be a useful therapeutic tool for increasing receptor expression and activity. We found that mGluR3 and mGluR2 are degraded robustly by the proteasome in both primary cells and a heterologous overexpression system. Additionally, we found that the proteasomal degradation of mGluR3 is ubiquitin-independent. Our data also indicated that mGluR2 and mGluR3 are

upregulated by cellular stress induced by dimethyl sulfoxide treatment. Lastly, we found that mGluR2 and mGluR3 signal through the AKT survival pathway, thereby supporting a role for the receptors in neuroprotection.

These findings provide novel insight into the activity and regulation of mGluR3 and will contribute important information to the field of schizophrenia research. Through an enhanced understanding of mGluR3 trafficking, signaling, and degradation, it will be possible to assess the role of the receptor in schizophrenia more closely and to design greatly needed therapeutic drugs.

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I. Introduction

Schizophrenia

Schizophrenia is a debilitating psychiatric disease that affects approximately 1% of the world's population (Freedman, 2003). Characteristics of schizophrenia include positive symptoms such as hallucinations, paranoia, and delusions, which may occur alone or in combination with negative symptoms such as lack of speech, flat affect, and attention impairment (Andreasen & Olsen, 1982). Symptoms of schizophrenia usually appear in late adolescence or early adulthood and remain present throughout the life of a patient (American Psychiatric Association, 1994). This chronic illness not only alters the lives of those who are afflicted, but also imposes a great burden on the healthcare systems of nations around the world. As of 2002, the total cost of schizophrenia to the United States due to factors such as excess healthcare costs and unemployment was $62.7 billion (Wu et al., 2005).

The causes of schizophrenia are still largely unknown, although it is clear that there is a moderate genetic component to the illness (Gejman et al., 2010; Gejman et al., 2011). Genome-wide association studies (Bergen & Petryshen, 2012; Lee et al., 2012) and twin studies have shown that schizophrenia is at least partially heritable (Sullivan et al., 2003). However, genetic factors cannot explain many cases of schizophrenia, and it is clear that environment plays a significant role as well. Factors such as living in an urban environment (Krabbendam & van Os, 2005), exposure to drugs including cannabis (Moore et al., 2007), and maternal infection during pregnancy (Brown, 2011; Sorensen et al., 2009) can all contribute toward increased risk for developing schizophrenia (van Os & Kapur, 2009).

There is no cure for schizophrenia, but the disease can be managed to varying degrees. Antipsychotic medications are often given and may be used in conjunction with psychosocial treatments such as cognitive behavioral therapy and extended rehabilitation. Unfortunately, antipsychotic treatment is only successful in about half of patients, with the other half being classified as treatment-resistant (Kane, 1996; Quintero et al., 2011). Antipsychotics are known to cause a variety of adverse effects, such as tardive dyskinesia (Margolese et al., 2005; Tenback & van Harten, 2011), weight gain (Allison et al., 1999), fatal cardiac symptoms (Ray et al., 2009), and hormonal fluctuations (Bostwick et al., 2009) in a shockingly high percentage of patients (Muench & Hamer, 2010; Tandon, 2011). Due to a high incidence of very unpleasant side effects, many schizophrenic patients do not adhere properly to medication schedules (Dibonaventura et al., 2012). Clearly, there is a great need for the development of new, safer, and more effective medications that will provide relief to people suffering from schizophrenia.

The Dopamine Hypothesis of Schizophrenia

For many decades it has been believed that schizophrenia stems from dysregulation of the dopamine system in the brain. The dopamine hypothesis of schizophrenia became popular only after the first medications for schizophrenia were accidentally developed. The first class of antipsychotics was discovered by chance in the mid-1950s. At that time chlorpromazine, a drug that was initially designed as a surgical anesthetic, was found to be effective at controlling symptoms in psychiatric patients (Pieters & Majerus, 2011). Several similar drugs followed shortly after

chlorpromazine and although these medications were oddly helpful for psychiatric patients, their mechanisms of action were unknown for many years. It was later discovered that these early neuroleptics acted by inhibiting dopamine receptors (Seeman, 1987). During this same era, it was noted that abuse of psychostimulants such as amphetamines, which act in part by increasing the release of dopamine, could induce psychopathological states very difficult to distinguish from schizophrenia (Kokkinidis & Anisman, 1981). Based on these findings and many others, the dopamine hypothesis of schizophrenia was dominant for decades. Much pharmacological and biochemical data has been generated to support a significant role for dopamine dysregulation in schizophrenia.

While it is clear that dopamine plays a role in psychosis, it has become evident over recent years that dopamine dysregulation is not the sole factor involved in schizophrenia. Antipsychotics that target dopamine receptors have only moderate efficacy in a select number of patients and do not generally impact some of the most severe symptoms, including cognitive and emotional impairment (Paz et al., 2007). Due to the failure of dopaminergic drugs to serve as an adequate treatment, scientists have recently turned to other neurotransmitter systems for insight into the etiology and treatment of schizophrenia.

The Glutamate Hypothesis of Schizophrenia

There is now significant evidence that implicates the glutamate system in schizophrenia. Glutamate is the most abundant neurotransmitter in the mammalian brain, and it is known to be involved in cognitive functions such as learning, memory,

and emotion (McEntee & Crook, 1993). Initial studies indicated that patients with schizophrenia might have abnormally low levels of glutamate in the cerebrospinal fluid (Kim et al., 1980). Multiple markers of glutamatergic metabolism have also been shown to be abnormal in brains of untreated schizophrenic patients as compared to brains of those who received neuroleptic treatment, suggesting that glutamatergic dysfunction may be relevant for both disease etiology and treatment (Sherman et al., 1991; Tsai et al., 1995).

Significant support for the glutamate hypothesis of schizophrenia came when scientists discovered that the recreational drug phencyclidine (PCP), which produces symptoms very similar to those found in schizophrenia, acts as an antagonist for the N-methyl-d-aspartate (NMDA) ionotropic glutamate receptor (Olney & Farber, 1995; Olney et al., 1999). Other NMDA receptor antagonists, such as ketamine, also elicit symptoms characteristic of schizophrenia (Malhotra et al., 1996; Newcomer et al., 1999). Additional findings have shown a strong correlation between low NMDA receptor mRNA levels and cognitive deficits in schizophrenic patients (Hirsch et al., 1997).

Many scientists now agree that the complexities of schizophrenia cannot be explained by one neurotransmitter system alone; rather, the disease probably depends upon dysfunction in a wide range of systems, including glutamate, dopamine, acetylcholine (Tandon, 1999), serotonin (Meltzer et al., 2003), and others (Stone & Pilowsky, 2007). Importantly, there is extensive interaction between the glutamate and dopamine systems, and these interactions might explain the overlapping glutamate and dopamine hypotheses of schizophrenia (Kegeles et al., 2000; David et al., 2005; Laruelle et al., 2003). As the field of psychopharmacology progresses, it will be

important to continue to study glutamate receptors, dopamine receptors, and interactions between the two systems as they relate to schizophrenia etiology and treatment.

II. Background

Metabotropic Glutamate Receptor Three

Metabotropic glutamate receptor three (mGluR3) has emerged as one of the most important elements in the glutamate hypothesis of schizophrenia. All metabotropic glutamate receptors are G protein-coupled receptors (GPCRs), meaning that they span the cellular membrane seven times and initiate intracellular signaling through bound G proteins following extracellular ligand binding. Briefly, ligand binding induces a conformational change in a GPCR, leading to activation of its associated heterotrimeric G protein via exchange of GDP for GTP. The heterotrimeric G protein consists of α, β, and γ subunits. Upon activation, the GTP-bound α subunit dissociates from the β and γ subunits, and the α subunit and βγ complex can both go on to affect intracellular signaling proteins (Hamm, 1998; Wettschureck & Offermanns, 2005). Different subtypes of G protein α subunits have different intracellular signaling targets (Simon et al., 1991; Hepler & Gilman, 1992; Neves et al., 2002). GPCRs are involved in many diseases, and recent estimates report that at least 30% of all prescribed drugs target GPCRs (Gruber et al., 2010; Kontoyianni & Liu, 2012).

Metabotropic glutamate receptors are classified as class C (or class 3) GPCRs based on sequence homology and functional similarity (Foord et al., 2005). Within the mGluR family, receptors are divided into groups I, II, and III based on structure and

activity (Nakanishi, 1992; Conn & Pin, 1997). Metabotropic glutamate receptor three is a member of group II, along with its closely related protein mGluR2 (Nakanishi et al., 1994). Many studies have found that mGluR3 and mGluR2 are coupled to $G_{\alpha i}$ and $G_{\alpha o}$, as their stimulation leads to inhibition of cyclic AMP formation by adenylate cyclase (Tanabe et al., 1993; Chavis et al., 1994). Furthermore, mGluR3 and the other $G_{\alpha i}$-coupled mGluRs are thought to be negatively coupled to the NMDA receptor (Ambrosini et al., 1995).

Messenger RNA encoding mGluR3 can be found in both glial cells and neurons in many regions of the rat brain, including the cerebral cortex, dentate gyrus, olfactory nucleus, basal ganglia, and cerebellum (Ohishi et al., 1993). There appear to be minimal species differences in mGluR3 distribution, as mRNA has been found in the human cerebral cortex, dentate gyrus, thalamus, and cerebellum (Makoff et al., 1996). The distribution of mGluR3 within neurons is primarily presynaptic (Petralia et al., 1996; Shigemoto et al., 1997). Although mGluR3 is approximately 100 kilodaltons in size, it tends to run on a polyacrylamide gel at both 100 kDa and, often more prominently, at or above 200 kDa, representing a higher-order species that may be a dimer (Blumcke et al., 1996).

In general, mGluR3 and mGluR2 are extremely similar, and most commercially available drugs and antibodies do not select for one receptor over the other (Harrison et al., 2008). Knockout mice for both receptors have been generated, and neither strain is grossly abnormal in terms of anatomy or function, perhaps due to compensation by the other receptor (Yokoi et al., 1996; Linden et al., 2005). Double mGluR2/mGluR3 knockout mice have recently been developed, and preliminary studies have shown that

they exhibit impaired spatial hippocampal learning and locomotor hypoactivity (Lyon et al., 2011).

Multiple studies have shown that group II mGluRs are neuroprotective (Pizzi et al., 1996; Bruno et al., 1998). Early studies in animal models have also shown that group II mGluRs are involved in anxiety and psychosis. For instance, a group II mGluR agonist has been shown to be effective at alleviating motor disturbances induced by the application of psychoactive drugs in rats (Cartmell et al., 2000). Agonists of group II mGluRs are also known to produce anxiolytic effects in certain models (Tatarczynska et al., 2001). Additionally, activation of group II mGluRs is thought to be helpful in alleviating neuropathic and inflammatory pain (Chiechio et al., 2004). Given these data, group II mGluRs have risen to the forefront of pharmacological studies, as they have the potential to serve as drug targets for a variety of diseases and conditions.

Connections between mGluR3 and Schizophrenia

A connection between group II mGluRs and schizophrenia was first made in 1998, when it was discovered that a group II mGluR agonist can reverse the behavioral effects of PCP administration in rats (Moghaddam & Adams, 1998). Since then genetic, expression, and pharmacological studies have all contributed to a growing hypothesis that mGluR3 is involved in schizophrenia etiology and may also be relevant for treatment. Since mGluR3 and mGluR2 are very similar, however, it has been difficult to differentiate between the two receptors in disease-related studies, so there is some dispute within the field regarding whether one or both receptors are involved in the disease.

Multiple independent genetic studies have found that certain polymorphisms within the GRM3 gene encoding mGluR3 are associated with cognitive impairment and heightened risk for schizophrenia in several families and populations (Egan et al., 2004; Chen et al., 2005; Baune et al., 2010). Other studies have shown that GRM3 polymorphisms may predict treatment outcomes with typical antipsychotics (Bishop et al., 2005). However, genetic linkage between mGluR3 and schizophrenia is a topic of great controversy, as several studies have found no association at all (Norton et al., 2005; Tochigi et al., 2006). Additionally, most polymorphisms that have been implicated in schizophrenia have either been synonymous or located in noncoding regions. Enough positive genetic data has been gathered that there still may be an association between GRM3 and schizophrenia, but more studies in larger populations are needed (Moreno et al., 2009).

Most studies have found no association between mGluR3 mRNA levels and schizophrenia (Ohnuma et al., 1998; Moreno et al., 2009). Although results of studies on mGluR3 protein levels have varied, there is significant evidence suggesting that mGluR3 protein may be altered in schizophrenic brains (Ghose et al., 2009). Importantly, one recent postmortem study showed a significant decrease in levels of mGluR3 dimeric/oligomeric forms in the prefrontal cortices of schizophrenic brains (Corti et al., 2007). These data fit with the trend of detection of mGluR3 protein in a 200 kDa or higher band, and also with hypotheses that the functional forms of many GPCRs are homodimeric or heterodimeric (Milligan, 2004; Minneman, 2007). Notably, due to the lack of alteration in mGluR3 mRNA levels but the possible deficiency of mGluR3 protein

in disease, disruption in expression is likely to occur at the level of protein stability, rather than transcription.

There is strong pharmacological evidence supporting the role of group II mGluRs in schizophrenia. Numerous studies have shown that mGluR2/3 agonists are successful at alleviating symptoms of schizophrenia induced by PCP in rodents (Cartmell et al., 2000; Chavez-Noriega et al., 2002). A small phase II clinical trial in humans also found that a group II mGluR agonist is more effective than placebo and equal in efficacy to the atypical antipsychotic olanzapine at alleviating both positive and negative symptoms of schizophrenia (Patil et al., 2007). Furthermore, this study suggested that a group II mGluR agonist might have lower rates of side effects than the established antipsychotic. This is the first instance of a drug that does not target a dopamine receptor being effective in treating symptoms of schizophrenia. However, it is still not clear whether group II mGluRs act alone in their control of schizophrenia, or whether they exert effects through the dopamine system, as group II mGluRs are known to regulate dopamine release (Greenslade & Mitchell, 2004; Chaki et al., 2006).

It is clear that group II mGluRs, and particularly mGluR3, may play a role in schizophrenia etiology and treatment. Although some controversy exists in the association between mGluR3 and disease, there is a large body of evidence that supports a connection, and poignant pharmacological studies highlight the relevance of this possible association. It will be necessary to gain more insight into the molecular mechanisms of mGluR3 action and regulation in order to better understand the role that it may play in one of the most serious mental illnesses. To this end, studies conducted in this project have aimed to elucidate the basic trafficking and regulatory mechanisms

of mGluR3. Drawing upon evidence suggesting that levels of mGluR3 protein may be abnormally low in schizophrenia, particular focus was given to mechanisms of mGluR3 degradation and methods of altering mGluR3 stability.

III. Materials and Methods

Degradation Pathway Assessment

There are two primary protein degradation pathways within eukaryotic cells: lysosomal degradation and proteasomal degradation. In general, proteasomal degradation is specific, while degradation by proteases contained within lysosomes is uncontrolled (Bohley, 1995). Since many diseases may be related to abnormal protein expression or stability, molecules involved in protein degradation pathways are excellent targets for newly developed therapeutics. No information has been published on mGluR3 degradation. The goal of this study was to determine whether mGluR3 is degraded by the proteasome and/or the lysosome within cells. This question was directed at understanding how mGluR3 is degraded in order to better grasp potential ways to manipulate levels of the protein in disease.

Human embryonic kidney (HEK-293T) cells were grown in complete DMEM High Glucose GlutaMAX medium (Invitrogen) containing 10% Fetal Bovine Serum (FBS, Atlanta Biologicals) and 1% Penicillin-Streptomycin (Invitrogen). Upon reaching 70% to 90% confluence, cells were transfected with cDNA encoding rat mGluR3 using Lipofectamine 2000 transfection reagent, according to instructions (Invitrogen). Cells from a single transfection were split and treated with vehicle, 50 μM leupeptin trifluoroacetate (Sigma), or 10 μM MG132 (Tocris). Leupeptin is a naturally occurring

protease inhibitor that specifically inhibits several proteases contained within lysosomes and effectively eliminates lysosomal degradation. Proteasomal degradation is potently and reversibly inhibited by MG132.

Following drug treatments, cells were harvested in harvest buffer containing 50 mM NaCl, 10 mM Hepes, 5 mM EDTA, 1 protease inhibitor tablet (Roche Diagnostics) and a small amount of Benzamidine Hydrochloride Hydrate (Fisher Scientific) per 50 mL of total buffer volume. Whole cell lysates were homogenized via membrane sonication, and total protein was normalized via a Bradford protein quantification assay. Samples were subjected to SDS-PAGE electrophoresis on Novex 4-20% Tris-Glycine gels (Invitrogen), and proteins were transferred to nitrocellulose at 30 volts for 120 minutes. Detection of mGluR3 was assessed and normalized actin was confirmed through Western blot.

Western Blot

Western blots were performed in a blocking buffer containing 2% nonfat dried milk, 50 mM NaCl, 10 mM Hepes, and 0.1% Tween 20 (Sigma). Primary antibodies used were polyclonal rabbit anti-mGluR2/3 (1:2000, Cell Signaling), polyclonal rabbit anti-actin (1:5000, Sigma), monoclonal rat anti-HA 3F10 (1:2500, Roche), monoclonal mouse anti-β-arrestin1 (1:2500, BD Biosciences), and monoclonal rabbit anti-β-arrestin2 (1:2500, Cell Signaling). Nitrocellulose membranes were incubated with primary antibodies for 1 hour. Secondary antibodies were Enhanced Chemiluminescence Horse Radish Peroxidase linked anti-mouse, anti-rat, and anti-rabbit (1:4000, GE Healthcare). Membranes were incubated with secondary antibodies

for 30 minutes. Blots were developed with Supersignal West Pico or ELISA Pico (Thermo Scientific).

Site-Directed Mutagenesis

In order to assess the effects of direct ubiquitination on mGluR3 degradation, a mutant version of mGluR3 that theoretically could not be ubiquitinated was created. Since ubiquitin is known to attach to lysine residues, all cytoplasmic lysine residues in mGluR3 were mutated to the similarly charged amino acid arginine. Eight point mutations were made using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). Mutations were confirmed with DNA sequencing, and the resulting mutant was called mGluR3-ZIK (Zero Intracellular Lysine [K]).

Degradation of mGluR3-ZIK versus mGluR3

Degradation pathways of mGluR3 and mGluR3-ZIK were compared in order to assess the effects of mutating candidate ubiquitination residues on mGluR3 degradation mechanisms. Both receptors were expressed in HEK-293T cells and degradation pathways were assessed as described above. Western blot was used to detect mGluR3 protein levels under conditions of treatment with different degradation inhibitors.

Co-Immunoprecipitation of mGluR3 and mGluR3-ZIK with HA-Ubiquitin

To further determine whether or not mGluR3 is directly ubiquitinated, co-immunoprecipitation studies were performed. HEK-293T cells were transfected to express either HA-tagged ubiquitin alone, HA-ubiquitin plus mGluR3, or HA-ubiquitin

plus mGluR3-ZIK. Between 24 and 48 hours after transfection, cells were harvested in a lysis buffer containing 50 mM NaCl, 10 mM Hepes, 5 mM EDTA, 1% Triton X-100 (Fisher Scientific), 1 protease inhibitor tablet, and a small amount of Benzamidine Hydrochloride Hydrate per 50 mL of total buffer volume. Samples were spun down and a small amount of soluble lysate was reserved. Meanwhile, protein A/G agarose beads (Pierce) were incubated with mGluR2/3 antibody for 30 minutes to facilitate attachment of the antibody to the beads. Remaining soluble lysates were removed from membrane fractions and incubated with the anti-mGluR2/3 beads end-over-end for 2 hours at 4°C. Next, beads were washed 3 times in lysis buffer and immunoprecipitated proteins were eluted off of the beads overnight in 2x sample buffer. Soluble lysate samples were assessed for total mGluR3, mGluR3-ZIK, and HA-ubiquitin expression. IP samples were analyzed to confirm immunoprecipitation of mGluR3 and mGluR3-ZIK and to investigate co-immunoprecipitation of HA-ubiquitin with the receptors.

Co-Immunoprecipitation of mGluR3 with β-arrestins

Small proteins known as β-arrestin1 and β-arrestin2 are known to be important in desensitization, internalization, degradation, and altered signaling of many GPCRs (Luttrell & Lefkowitz, 2002). One group found that co-overexpression of β-arrestin1 with mGluR3 in HEK-293T cells resulted in significant effects on cAMP and extracellular signal-regulated kinase (ERK) signaling outputs (Iacovelli et al., 2009). However, it was not previously known if mGluR3 interacts directly with the β-arrestin proteins and how this possible interaction might impact mGluR3 regulation.

Cells were transfected with cDNA encoding mGluR3 alone, HA-β-arrestin1 plus mGluR3, or HA-β-arrestin2 plus mGluR3. A co-immunoprecipitation assay was performed between 24 and 48 hours after transfection. Cells were washed in phosphate buffered saline with calcium (PBS/Ca^{2+}, Gibco), then incubated with 2 mM Dithiobis [succinimidyl propionate] (DSP, Thermo Scientific) for 30 minutes at room temperature in order to facilitate cross-linkage of protein-protein interactions. A standard immunoprecipitation reaction was performed as detailed above, except that HA-tagged β-arrestins were pulled down with monoclonal mouse anti-HA agarose beads (Sigma). Soluble lysate and immunoprecipitation samples were run on a gel and co-immunoprecipitation of mGluR3 with the immunoprecipitated β-arrestins was assessed via Western blot.

Comparison of Degradation Pathways of mGluR2 and mGluR3

Due to the fact that most therapeutically relevant drugs and commercially available antibodies target both mGluR2 and mGluR3, and due to evidence that both receptors may be involved in schizophrenia pathology and/or treatment, degradation pathways were generalized to both group II mGluRs. HEK-293T cells were transfected with cDNA encoding mGluR3 or mGluR2, then exposed to 10 μM MG132 beginning approximately 24 hours after transfection. The effects of a group II mGluR agonist on protein degradation were also assessed. Cells were treated with 10 μM LY354740 (Tocris) alone or concurrently with MG132 treatment in order to find out if activation of group II mGluRs alters the amount of attempted proteasomal degradation that occurs. Cells were harvested in harvest buffer without triton and protein concentrations were normalized before samples were run on a gel.

Assessment of Group II mGluR Degradation in Primary Cortical Rat Neurons

Primary neurons were cultured in order to confirm group II mGluR degradation patterns in native cells. Pregnant Sprague Dawley rats were obtained from Charles River and euthanized with carbon dioxide at embryonic day 18 to 20. Cortices from pups were dissected out and incubated in Papaine (Worthington) dissolved in Minimal Essential Medium (MEM, Invitrogen) for 1 hour. The enzymatic digest was halted by washing with MEM containing 10% dialyzed FBS and 1 µg per 100 mL DNase (Sigma). Cells were then mechanically dissociated with a fine-tipped glass pipette tip until the cell stock appeared homogenous. Cells were spun out and resuspended in MEM containing 10% dFBS, 1% Penicillin-Streptomycin, and 1 µg per 1 mL Fungizone (Invitrogen). Cells were plated on Poly-D-Lysine coated plates (Becton Dickinson). After approximately 24 hours cultures were switched to a serum-free medium containing Neurobasal medium (Invitrogen), 2% B-27 supplement (Invitrogen), 1% Penicillin-Streptomycin, 1% Glutamax 100X (Invitrogen), and 1 µg per 1 mL Fungizone. Half of the total volume of serum-free medium was changed every 3 to 4 days thereafter.

Cells were treated with 0.2% DMSO (vehicle, Sigma), 20 µM MG132, or 20 µM MG132 plus 10 µM LY354740 for long or short time periods following 7 to 9 days in culture. Cells were harvested, protein concentrations were normalized via a Bradford assay, and samples were assessed via Western blot as described above for experiments in HEK-293T cells.

Assessment of Group II mGluR Signaling in Primary Neurons

Multiple investigators have recently found that group II mGluRs may signal through the AKT survival pathway. An increase in phosphorylated AKT (pAKT) has been found following group II mGluR agonist treatment in the prefrontal cortex (Sutton & Rushlow, 2011), in astrocyte cultures (Durand et al., 2011) and in mixed neuron-astrocyte cultures (D'Onofrio et al., 2001). To our knowledge, AKT signaling has not been confirmed specifically in cortical neuronal cultures. Activation of AKT via phosphorylation is known to lead to a number of downstream effects including promotion of cell survival and overcoming of cell cycle arrest. Hence, it is necessary to fully characterize the AKT signaling output of group II mGluRs, as they are thought to be involved in neuroprotection.

Embryonic rat cortical neuron cultures were generated as described above. After 7 to 9 days in culture, cells were starved in Neurobasal medium without supplementation for approximately three hours in order to eliminate basal pAKT activation via growth factors contained in the medium. Cells were then stimulated with the group II mGluR agonist LY354740 at a 1 μM final concentration for 10 minutes. Additionally, opti-MEM (Invitrogen) was used as a positive control to stimulate pAKT through insulin and verify the efficacy of the assay. Following treatment, the medium was immediately aspirated and cells were harvested in 2X sample buffer containing Halt protease/phosphatase inhibitor cocktail (Pierce). Samples were homogenized via sonication and subjected to electrophoresis. Following transfer to nitrocellulose, membranes were blocked in Odyssey blocking buffer (Licor) for 30 minutes at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C in a solution containing one part Odyssey buffer and one part phosphate buffered saline

(PBS, Sigma) with 0.1% Tween 20. Primary antibodies were monoclonal mouse anti-pERK (1:500, Santa Cruz), polyclonal rabbit anti-total ERK (1:1000, Cell Signaling), monoclonal rabbit anti-pAKT at serine 473 (1:2000, Cell Signaling), and monoclonal mouse anti-total AKT (1:2000, Cell Signaling). Membranes were washed in PBS/Tween and incubated with secondary antibodies for 30 minutes at room temperature. Secondary antibodies were Alexa fluorophore 680 anti-rabbit (1:20,000, Invitrogen) and Licor fluorophore 800 anti-mouse (1:20,000, Licor). Fluorescence was detected and quantified using an Odyssey imaging machine with Image Studio software (Licor). Statistical analyses were performed using Prism software. All treatment conditions were performed in duplicate, and values were calculated as the average of the pAKT signal divided by the total AKT signal for both samples in each duplicate. Fold increase in the pAKT to total AKT ratio was calculated in comparison to vehicle treatment. AKT assays were also conducted following 0.2% DMSO (vehicle) or 20 μM MG132 pre-treatment of 5 to 12 hours.

Evaluation of mGluR3 Surface Expression via Biotinylation

Surface expression of mGluR3 was assessed in HEK-293T cells following treatment with vehicle or 10 μM MG132 in order to assess the effects of proteasomal inhibition on the amount of mGluR3 at the cell surface. Cells were transfected with cDNA encoding mGluR3 and the assay was conducted between 24 and 48 hours after transfection. Cells were treated with 0.2% DMSO (vehicle) or 10 μM MG132 for 10 to 12 hours. Cells were then washed in PBS/Ca^{2+} and treated with the biotinylation reagent EZ-link Sulfo-NHS-SS-Biotin (Thermo Scientific) at a 2 mM concentration in PBS/Ca^{2+}

for 30 minutes at room temperature. This reagent attaches a biotin group with a linker arm to all free extracellular amines, so it should label all proteins that traffic to the cell surface at some point during the biotinylation incubation. The biotinylation reaction was quenched by washing with 100 mM glycine (Sigma). Cells were then harvested in harvest buffer containing triton as described for co-immunoprecipitation experiments above. After spinning down samples, a portion of the soluble lysate was reserved for analysis and the rest was incubated with streptavidin agarose beads (Thermo Scientific) for 2 hours in order to pull down biotinylated molecules. Meanwhile, the membrane fraction was homogenized in 1x sample buffer via sonication. Following the pull-down, the streptavidin beads were washed 3 times in harvest buffer to reduce nonspecific binding. Biotinylated proteins were eluted off of the beads overnight in 2x sample buffer. Samples were subjected to electrophoresis and membrane, soluble lysate, and pull-down samples were blotted to evaluate mGluR3 solubility and surface expression.

Evaluation of Cycloheximide Effects on Group II mGluR Detection

After finding that both MG132 and DMSO alone can act by increasing the expression of group II mGluRs in certain types of cells, experiments were performed that attempted to identify the source of these expression changes. The translation inhibitor cycloheximide was used in an effort to separate effects on transcription from effects on stability in neurons treated with either DMSO or MG132. Theoretically, if DMSO impacts mGluR3 levels by upregulating transcription, then cycloheximide should abolish the effects of DMSO, while if MG132 impacts mGluR3 levels by inhibiting degradation, then cycloheximide should not alter the effects of MG132 as much.

Primary embryonic cortical rat neurons received treatments of cycloheximide (100 µg per 1 mL, Sigma), 20 µM MG132 and/or 0.2% DMSO for short and long periods of time. Cells were harvested in a harvest buffer without triton and protein concentrations were normalized with a Bradford assay. Samples were then run on a gel and Western blot was used to detect mGluR2/3 and actin.

IV. Results and Discussion

Degradation of mGluR3 Occurs through the Proteasome in HEK-293T Cells

In HEK-293T cells heterologously expressing mGluR3, significant accumulation of the receptor occurred following MG132 treatment, but only a small accumulation occurred following leupeptin treatment (Figure 1). Considerable cell death occurred following long periods of MG132 treatment, while moderate cell death occurred after leupeptin treatment. Detection of mGluR3 increased with longer MG132 treatments, showing that the proteasome may have been more efficiently inhibited and more attempts at receptor degradation may have occurred in those conditions. These results show that mGluR3 is degraded primarily by the proteasome.

Proteasomal Degradation of mGluR3 is Ubiquitin-Independent

Most proteins that are degraded by the proteasome are targeted to the proteasome by poly-ubiquitination of lysine residues. For transmembrane receptors, ubiquitination occurs at lysines that are in the cytoplasm while the receptor is in the cell membrane (Hershko & Ciechanover, 1998). In order to find out if the proteasomal degradation of mGluR3 is ubiquitin-dependent, a mutant version of mGluR3 with no

intracellular lysine residues was created. Previous studies have indicated that for another mGluR, mGluR5, all intracellular lysines had to be mutated before an effect on ubiquitination could be found (Moriyoshi et al., 2004). Although some proteins have a specific known lysine that is ubiquitinated, many receptors require mutation of all intracellular lysines in order to halt ubiquitination (Saur et al., 2010; Giordano et al., 2011).

Site-directed mutagenesis was successfully used to mutate all intracellular lysine residues of rat mGluR3, resulting in the mutant mGluR3-ZIK (Figure 2). Surprisingly, mGluR3-ZIK exhibited nearly identical degradation patterns as wild-type mGluR3 in a heterologous system in HEK-293T cells. Both receptors accumulated equivalently with increasing length of exposure to MG132 (Figure 3). Additionally, both receptors exhibited the same lack of accumulation with leupeptin treatment (data not shown). These results were surprising, as elimination of ubiquitination sites should prevent proteasomal degradation for most proteins that undergo degradation by the classic ubiquitin-proteasome system.

Co-immunoprecipitation studies of mGluR3 and mGluR3-ZIK with HA-ubiquitin revealed that neither the mutant nor the wild-type receptor is ubiquitinated (Figure 4). There was no co-immunoprecipitation of HA-ubiquitin with mGluR3 or with mGluR3-ZIK. This provides further evidence that mGluR3 is not ubiquitinated, so its degradation by the proteasome must occur in a ubiquitin-independent manner. A lack of physical interaction between a protein and ubiquitin and a failure to notice changes in degradation patterns when all candidate lysines of the protein are mutated are two of

the major hallmarks of proteins that are degraded by the proteasome in a ubiquitin-independent fashion (Jariel-Encontre et al., 2008).

Although it was originally thought that ubiquitination was mandatory for proteasomal degradation, accumulating evidence suggests that many proteins are degraded by the proteasome without being ubiquitinated. It is now estimated that approximately 20% of cellular proteins are degraded by the proteasome independently of ubiquitin (Baugh et al., 2009). Many proteins that are degraded in this way have intrinsically disordered α-helical segments that are directly targeted by the proteasome (Baugh et al., 2009; Melo et al., 2011). Other proteins may be directed to the proteasome by small ubiquitin-like molecules that serve as a label for degradation (Jariel-Encontre et al., 2008). So far, few if any GPCRs have been reported as being degraded by the proteasome in a ubiquitin-independent fashion, so these data represent an especially noteworthy finding.

β-arrestins 1 and 2 Physically Interact with mGluR3 in a Cellular Context

Co-immunoprecipitation studies revealed novel interactions between mGluR3 and both β-arrestin proteins (Figure 5). These interactions may be important for mGluR3 desensitization, internalization, and degradation, since β-arrestins are known to regulate such activities in other GPCRs. Several attempts were made to knock down β-arrestins with siRNA in order to study the effect of β-arrestins on mGluR3 degradation and/or trafficking. However, a significant knockdown could not be achieved (data not shown). In the future it will be important to investigate the function of these novel interactions in terms of mGluR3 regulation.

Group II mGluRs are Degraded by the Proteasome in Primary Neurons

No distinction between mGluR2 and mGluR3 can be made in wild-type native cells due to a lack of specificity of the mGluR2/3 antibody. Thus, before assessing group II mGluR degradation in primary neurons, degradation pathways of the individual receptors were compared in HEK-293T cells. Both receptors accumulated identically with MG132 treatment, indicating that mGluR2, like mGluR3, is degraded by the proteasome in HEK-293T cells (Figure 6). There was no significant effect of agonist treatment on degradation of either receptor, suggesting that the group II mGluRs may be constitutively active, may be constantly stimulated by glutamate released into the medium, or may be degraded in a manner that is not regulated by receptor activity. It is important to note that the mGluR2/3 antibody detects mGluR2 and mGluR3 slightly differently, with enhanced detection of the mGluR2 monomer as opposed to the higher-order species. These data revealed that the effect of MG132 can be generalized to group II mGluRs, which is important for the assessment of native cells since no distinction can be made between the two receptors.

Cultured embryonic cortical rat neurons were treated with DMSO (vehicle) or MG132 for short and long periods of time. A significant accumulation, especially of the higher-order species, occurred with MG132 treatment. No significant change was observed when the group II mGluR agonist was added, indicating that the effect of MG132 may be agonist-independent. These data revealed for the first time that group II mGluRs are degraded by the proteasome in native cells (Figure 7). These results

supported the findings in HEK-293T cells and confirmed the degradation pathway of group II mGluRs.

Expression of Group II mGluRs is Upregulated by DMSO Treatment in Neurons

Overall expression of mGluR2/3 was massively increased after 24 hours of 0.2% DMSO treatment in comparison to shorter periods of treatment or no treatment at all (Figure 8). This is in sharp contrast to HEK-293T cells, in which DMSO application has no effect on mGluR2 or mGluR3 expression. DMSO is known to induce cellular stress and apoptosis (Vondracek et al., 2006; Hanslick et al., 2009). Group II mGluRs are also known to exhibit increased expression in conditions of cellular stress, such as inflammation (Berger et al., 2012). Therefore, it appears that group II mGluRs may be upregulated for purposes of neuroprotection when primary neurons are exposed to the stress induced by DMSO treatment. This does not occur in HEK-293T cells because they do not normally express group II mGluRs and therefore might not possess the factors that are necessary to upregulate endogenous mGluR expression. These findings further support the proposed role of group II mGluRs in neuroprotection and promotion of cell survival.

Activation of Group II mGluRs Leads to AKT Signaling in Primary Rat Neurons

The ratio of phosphorylated AKT to total AKT rose approximately 1.6-fold following 10 minutes of stimulation with 1 μM LY354740 compared to vehicle treatment in primary cortical embryonic rat neurons (Figure 9). These data showed that group II mGluRs signal through AKT in neurons. In contrast with data reported previously by

other groups, no pERK response was detected (data not shown). These data support the hypothesis that group II mGluRs are important in neuroprotection, as they signal through a survival pathway.

In order to determine the effects of increased group II mGluR expression on signaling, pAKT assays were performed following 5 to 12 hours of pre-treatment with DMSO or MG132. Since these reagents both increase detection of group II mGluRs in neurons, it is important to know whether or not the increased protein also correlates with increased functionality through signaling. However, these experiments could not be interpreted due to technical obstacles. The effects of DMSO and MG132 on group II mGluR expression are dramatically decreased when cells are starved for 3 hours before the pAKT assay (Figure 10). This may be because nutrient starvation can induce autophagy in many cell types, so proteins may undergo degradation by alternative pathways that are not physiologically relevant in cases of severe nutrient deprivation (Mizushima, 2007). The pAKT assay cannot be performed without starvation, as growth factors in the medium stimulate the pathway and result in such high background that even the positive control of opti-MEM has no effect over vehicle (data not shown). Additionally, MG132 treatment causes pAKT to accumulate to nearly the same extent as agonist treatment, so it is impossible to study the effects of the agonist in addition to MG132 due to this high background (data not shown). Therefore, it is not feasible to assess the effects of MG132 and DMSO on group II mGluR functionality through this pAKT assay.

A Higher-Order mGluR3 Species is Expressed on the Cell Surface, but Surface Biotinylation Cannot be Used to Evaluate the Effects of MG132

Biotinylation assays conducted in HEK-293T cells expressing mGluR3 revealed that there was no change in mGluR3 expression following MG132 treatment in the soluble lysate samples that were eventually used for the streptavidin pull-down (Figure 11a). Rather, the increased detection of mGluR3 under conditions of MG132 treatment that is characteristic of whole cell lysates was found only in the membrane samples (Figure 11b). This indicates that MG132 has a profound influence on the solubility of mGluR3, as it changed the fraction of the receptor population that was found in the soluble lysate versus membrane samples. Therefore, the effects of MG132 on surface expression cannot be evaluated through this assay, since the increase in mGluR3 that occurs after MG132 treatment is not represented in the soluble fraction that is incubated with the beads and consequently cannot be pulled down and identified. Note that there is also a large reduction in mGluR3 solubility with biotinylation treatment (Figure 11b). However, these experiments did show that the 250 kDa higher-order species of mGluR3 that is often detected in Western blot does indeed represent a population of receptors that are expressed at the surface, as proteins in this band were pulled down with the streptavidin beads, indicating that they were biotinylated at the surface of the cells (Figure 11c).

Cycloheximide Has Unexpected Effects on Group II mGluR Detection in Neurons

Cells that were treated with cycloheximide, either alone or in combination with DMSO or MG132, exhibited higher expression of group II mGluRs (Figure 12). This

result is counterintuitive, as cycloheximide is known to inhibit translation and result in decreased levels of protein over time. It is possible that cycloheximide was used at a concentration that was high enough to cause cellular stress, but not high enough to adequately inhibit ribosomes, so the treatment resulted in a neuroprotective increase of group II mGluR expression. To resolve this unexpected result, more trials will have to be performed using different concentrations of cycloheximide.

V. Conclusions

The results of this project have shown that group II mGluRs are degraded by the proteasome. These findings have been confirmed in both HEK-293T cells transfected to express mGluR2 or mGluR3 and in primary embryonic cortical rat neurons that express group II mGluRs endogenously. The elucidation of the degradation pathway of these receptors represents a novel finding that may be important for understanding how to manipulate group II mGluRs in disease. For instance, if there is a lack of mGluR3 in the brains of schizophrenic patients, it might be possible to create a drug targeting the degradation pathway that will increase the stability of mGluR3 in order to return it to normal levels of expression. Before such a drug can be created, it will first be necessary to elucidate the mechanism by which mGluR3 is degraded by the proteasome. This project has shown that mGluR3 is unexpectedly degraded in a ubiquitin-independent fashion, thereby eliminating elements involved in ubiquitination from the list of potential drug targets. The β-arrestin proteins may be involved in mGluR3 internalization and degradation, but more studies will need to be performed in order to determine the significance of the mGluR3 and β-arrestin interaction in terms of receptor regulation. For

instance, it may be helpful to manipulate β-arrestin levels or activity via a dominant-negative mutant (Krupnick et al., 1997) or knockout model, and then study the effects of β-arrestins on mGluR3 degradation pathways.

The finding that mGluR3 is degraded by the proteasome in a ubiquitin-independent manner was extremely unexpected. Although the number of proteins that are known to be degraded in this manner is growing, this type of degradation is still considered relatively unusual. Many of the proteins that are degraded by the proteasome without being ubiquitinated are involved in diseases such as neurodegeneration (David et al., 2002) and cancer (Zetter & Mangold, 2005), but few if any have been implicated directly in psychiatric disease. It will be necessary to study the ubiquitin-independent mechanism of mGluR3 degradation in greater detail and to discover the exact ways in which the receptor is targeted to the proteasome in order to make progress toward being able to create a drug that targets the degradation pathway of the receptor.

The significant effect of DMSO treatment on group II mGluR expression in primary neurons was a novel finding that may be important for both studying and targeting group II mGluRs in the future. It is often difficult to detect mGluR3 protein in astrocytes, so it may be useful for investigators who study mGluR3 in astrocytes to use DMSO to upregulate expression of the receptor. Additionally, by studying the mechanisms by which DMSO induces increased expression of group II mGluRs, it may be possible to develop drugs that will mimic the effect of DMSO on group II mGluR levels without causing cellular stress.

These results will be strengthened by functional data showing that DMSO and/or MG132 result not only in an increase in receptor expression, but an increase in functionality as well. It may be useful to experiment with adjusting the length of time of starvation for pAKT assays to find a middle point at which the effects of DMSO and MG132 are still detectable and the assay works. The AKT signaling pathway is likely to be very important, as evidence suggests that there is an association between low AKT signaling activity and schizophrenia (Emamian et al., 2004; Arguello & Gogos, 2008). Furthermore, many antipsychotic drugs result in an increase in AKT signaling (Freyberg et al., 2009), so the novel discovery that group II mGluRs signal through this pathway in neurons may give insight into the mechanisms by which group II mGluR agonists help alleviate symptoms of schizophrenia.

It will also be important to continue to investigate the effects of DMSO and/or MG132 on group II mGluR surface expression. Since the impact of MG132 on receptor solubility makes the surface biotinylation assay unusable, it may be constructive to use an N-terminal antibody for cell surface luminescence or to use radioligand binding in order to evaluate surface expression. Additionally, the biotinylation assay could potentially be more useful if the cells were solubilized in a harvest buffer containing a stronger detergent, as this might resolve the issue of decreased receptor solubility following MG132 treatment.

Overall, the data gained in this project have contributed insight into the ways that the expression of group II mGluRs, and especially mGluR3, is affected by degradation and cellular stress. These findings are significant, as they may lead to a better understanding of the ways in which mGluR3 can be manipulated as a treatment for

schizophrenia. As the connections between ubiquitin-independent proteasomal degradation, upregulation in conditions of cellular stress, signaling through AKT, and β-arrestin interactions are strengthened, doors toward the discovery of more effective antipsychotic drugs that target group II mGluRs will be opened.

VI. Figures

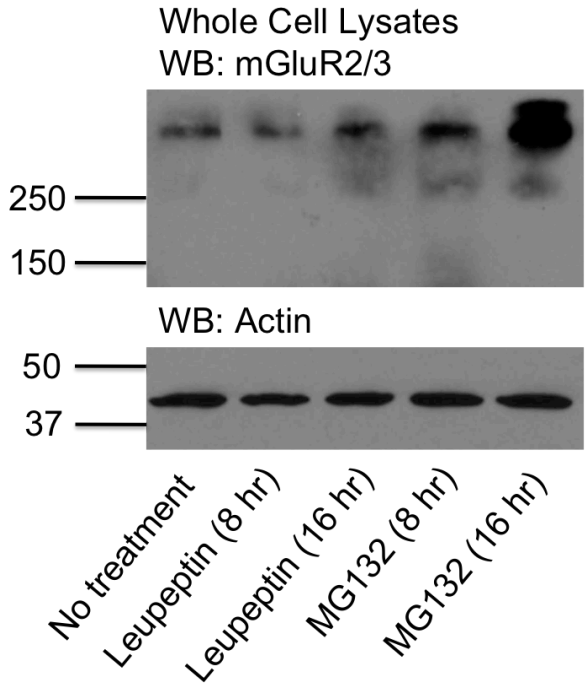

Figure 1: Proteasomal degradation of mGluR3. A representative Western blot of mGluR2/3 and actin under conditions of 50 μM leupeptin or 10 μM MG132 treatment is shown. Detection of mGluR3 is primarily at and above 250 kDa, representing a higher-order species that might be an oligomer.

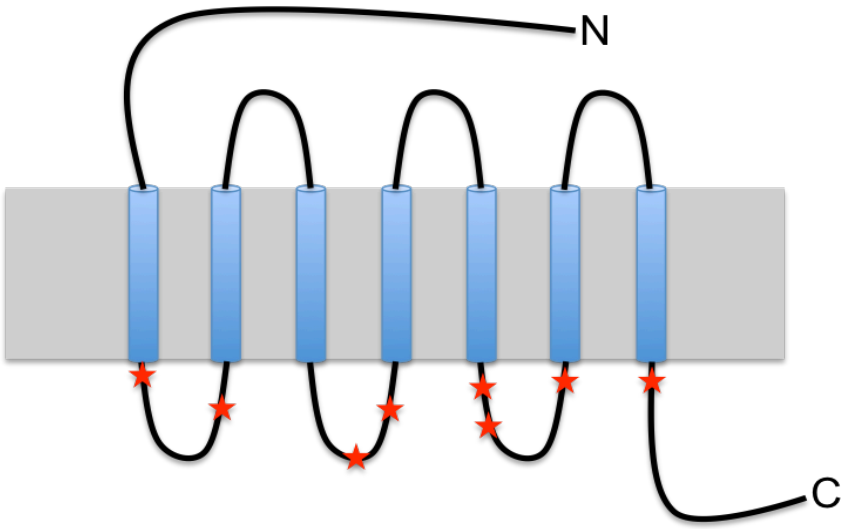

Figure 2: Mutated intracellular lysines in mGluR3-ZIK. Red stars indicate locations of lysine to arginine mutations. Single base substitutions were used to convert AAG or AAA to AGG or AGA. Mutations were verified through DNA sequencing and occurred at bases 1799, 1823, 2042, 2270, 2279, 2306, and 2495, representing mutations in amino acids 600, 608, 674, 681, 757, 760, 769, and 832.

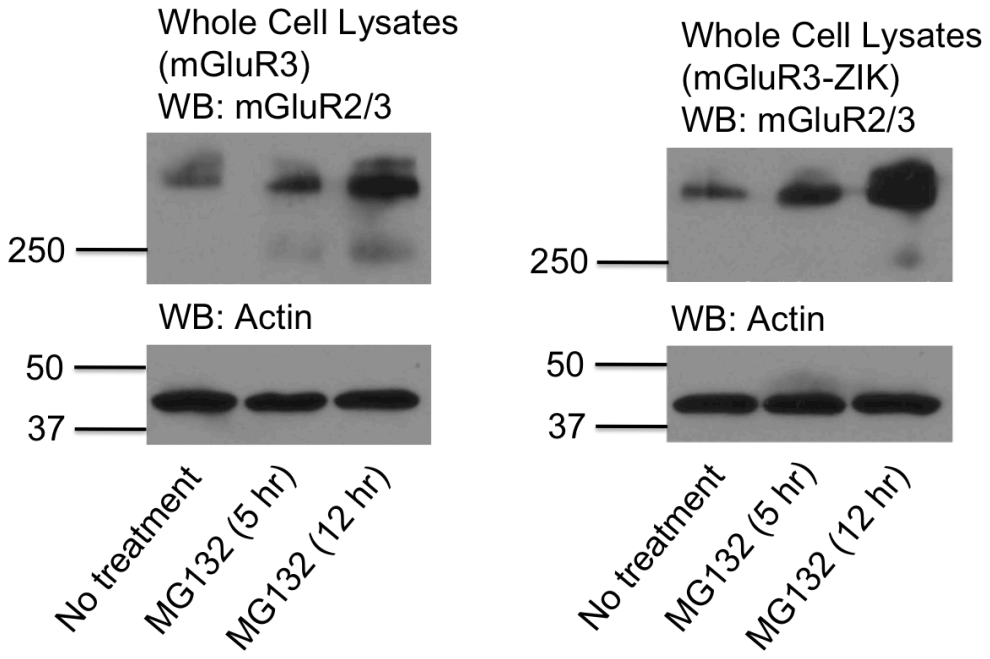

Figure 3: Equivalent proteasomal degradation of mGluR3 and mGluR3-ZIK indicates ubiquitin-independent degradation mechanism. Representative Western blots of mGluR2/3 and actin under conditions of 10 μM MG132 treatment are shown. Both receptors accumulate equivalently with proteasomal inhibition and are detected in the same higher-order bands.

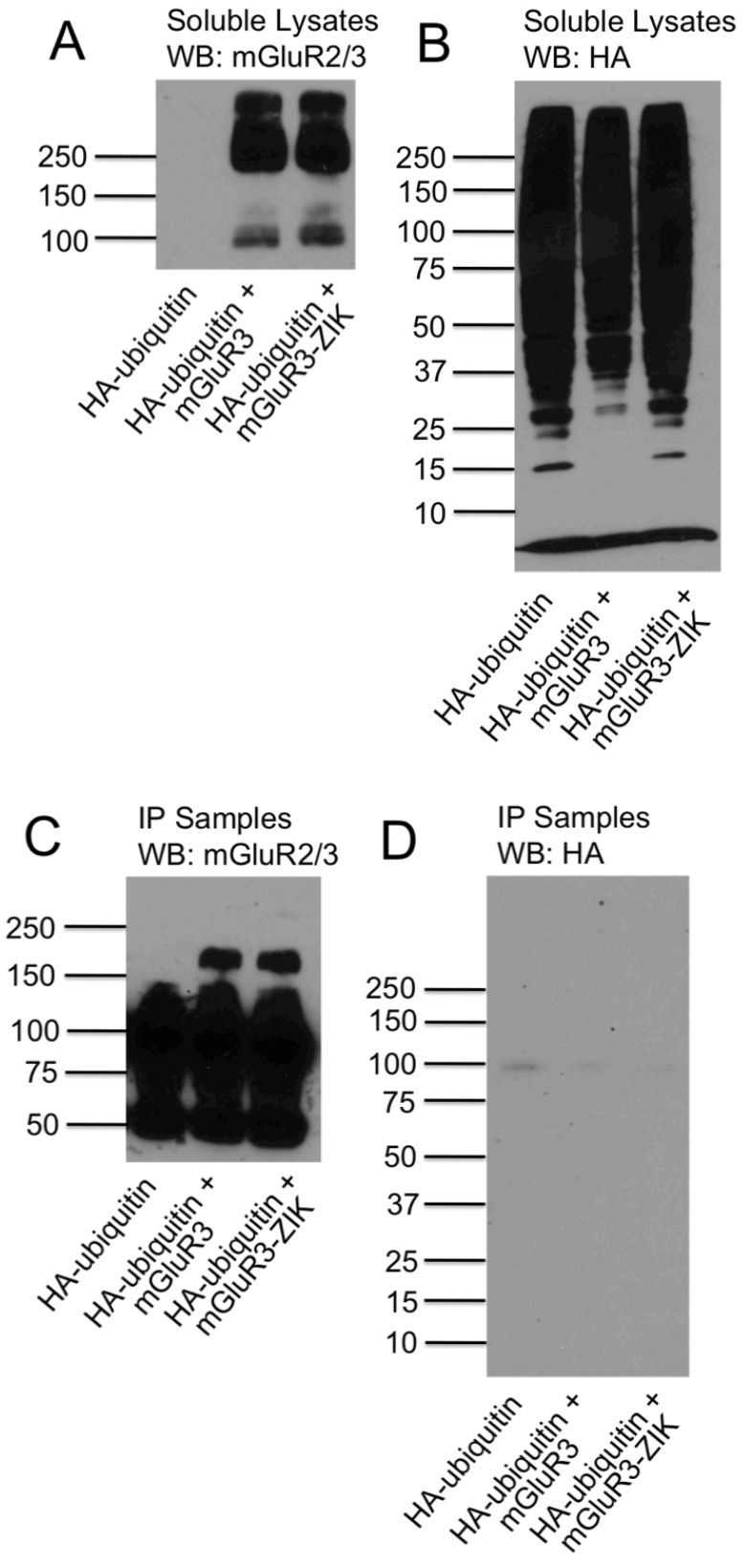
A
Soluble Lysates
WB: mGluR2/3
250
150
100
HA-ubiquitin
HA-ubiquitin + mGluR3
HA-ubiquitin + mGluR3-ZIK
B
Soluble Lysates
WB: HA
250
150
100
75
50
37
25
15
10
HA-ubiquitin
HA-ubiquitin + mGluR3
HA-ubiquitin + mGluR3-ZIK
C
IP Samples
WB: mGluR2/3
250
150
100
75
50
HA-ubiquitin
HA-ubiquitin + mGluR3
HA-ubiquitin + mGluR3-ZIK
D
IP Samples
WB: HA
250
150
100
75
50
37
25
15
10
HA-ubiquitin
HA-ubiquitin + mGluR3
HA-ubiquitin + mGluR3-ZIK

Figure 4: Metabotropic glutamate receptor three is not ubiquitinated. A) Both mGluR3 and mGluR3-ZIK show equal expression and banding patterns in the soluble lysates. B) HA-ubiquitin is robustly expressed in all conditions. C) The immunoprecipitation of mGluR3 and mGluR3-ZIK was successful. Note that smears between 50 kDa and 150 kDa represent antibody cross-reactivity due to the same antibody being used for the pull-down and the blot. D) HA-ubiquitin does not co-immunoprecipitate with mGluR3 or mGluR3-ZIK. A slight mark at 100 kDa in the HA-ubiquitin alone lane represents background of HA-ubiquitin sticking to the beads.

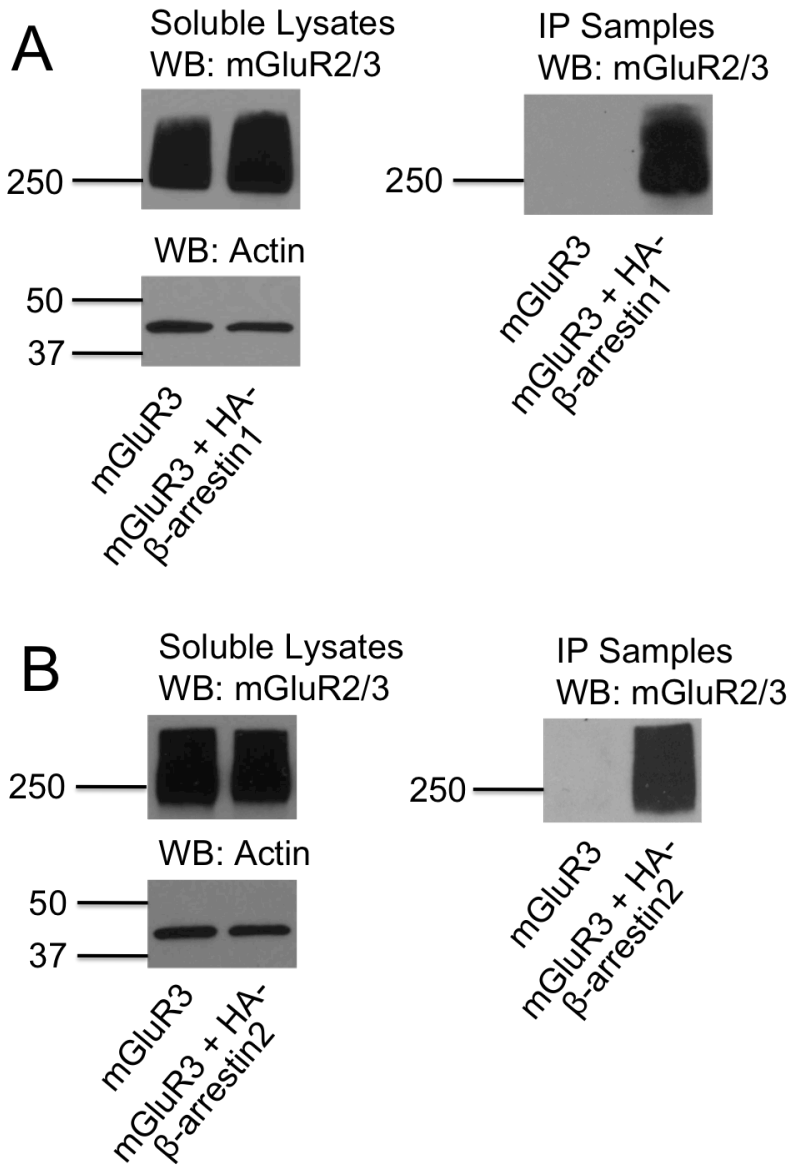

Figure 5: Both β-arrestin1 and β-arrestin2 interact with mGluR3 in a cellular context. Assays were performed following 30 minutes of cross-linking with 2 mM DSP. A) Soluble lysate samples show equal input of mGluR3 and immunoprecipitation samples show co-immunoprecipitation of mGluR3 with HA-β-arrestin1 with no background of mGluR3 sticking to the anti-HA beads. B) Soluble lysate samples show equal input of mGluR3 and immunoprecipitation samples show co-immunoprecipitation of mGluR3 with HA-β-arrestin2.

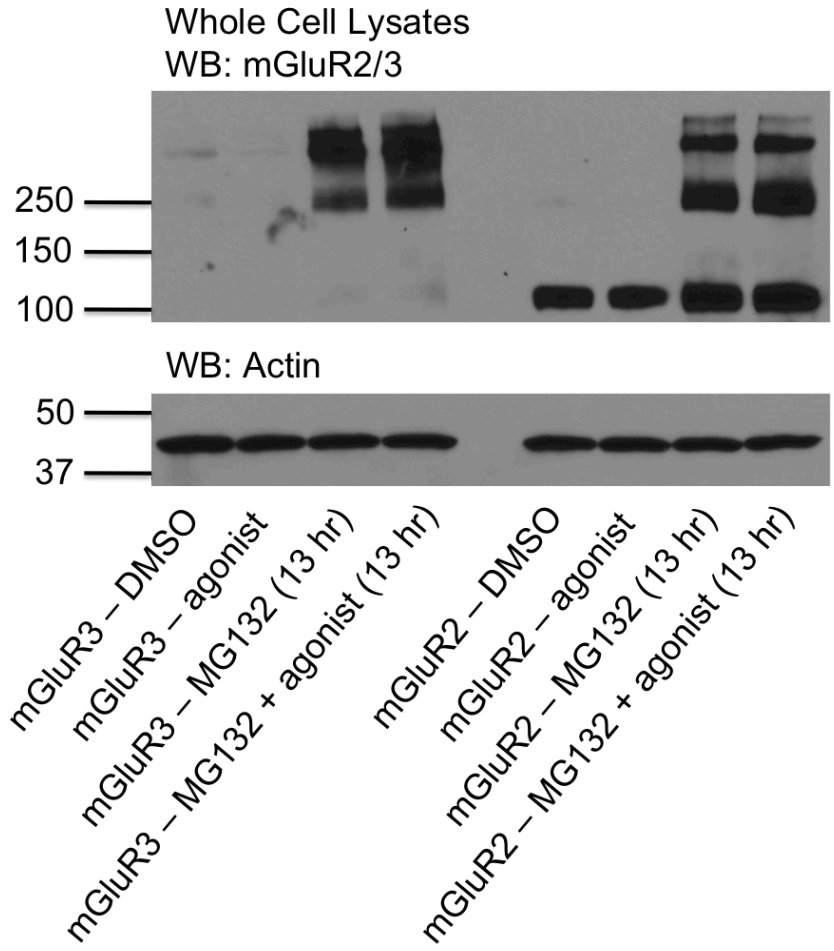

Figure 6: Both group II mGluRs undergo similar proteasomal degradation that is not agonist-dependent. A representative Western blot of mGluR2 and mGluR3 under conditions of 0.2% DMSO (vehicle), 10 μM LY354740 agonist, and/or 10 μM MG132 is shown. This blot shows a particularly robust increase in mGluR detection following MG132 treatment, but the increase is not substantially affected by agonist treatment.

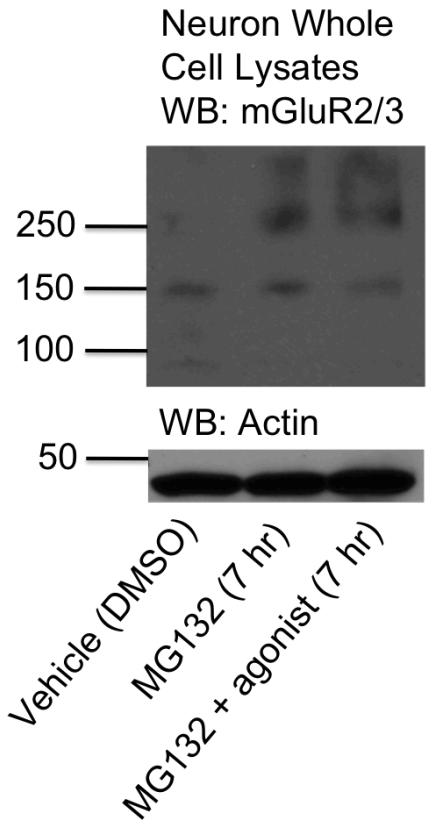

Figure 7: Group II mGluRs are degraded by the proteasome in primary cortical embryonic rat neurons. A representative blot of mGluR2/3 shows a significant accumulation with 20 μM MG132 treatment in comparison to vehicle, especially in the higher-order species. This effect is not impacted by the co-application of 10 μM LY354740.

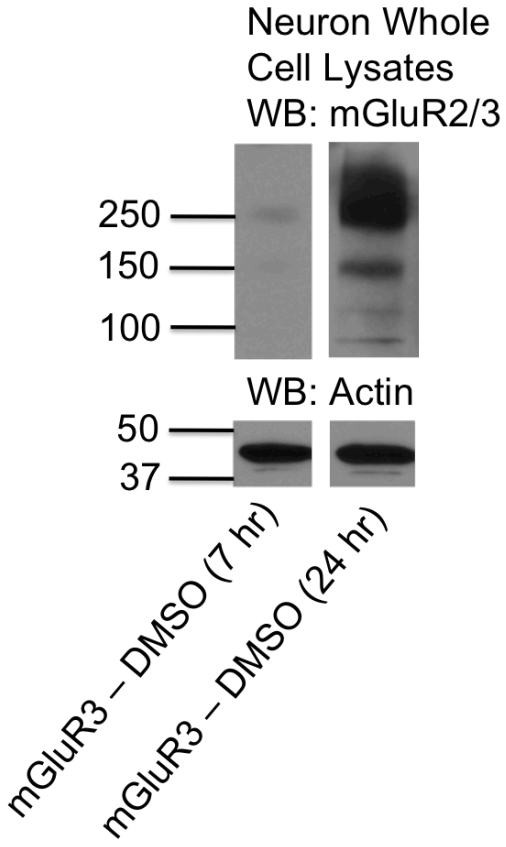

Figure 8: DMSO treatment results in a significant upregulation of group II mGluR expression in primary cortical embryonic rat neurons. A representative blot shows a larger increase in detection of group II mGluRs in both the monomer and higher-order bands following 0.2% DMSO treatment for 24 hours in comparison to 7 hours.

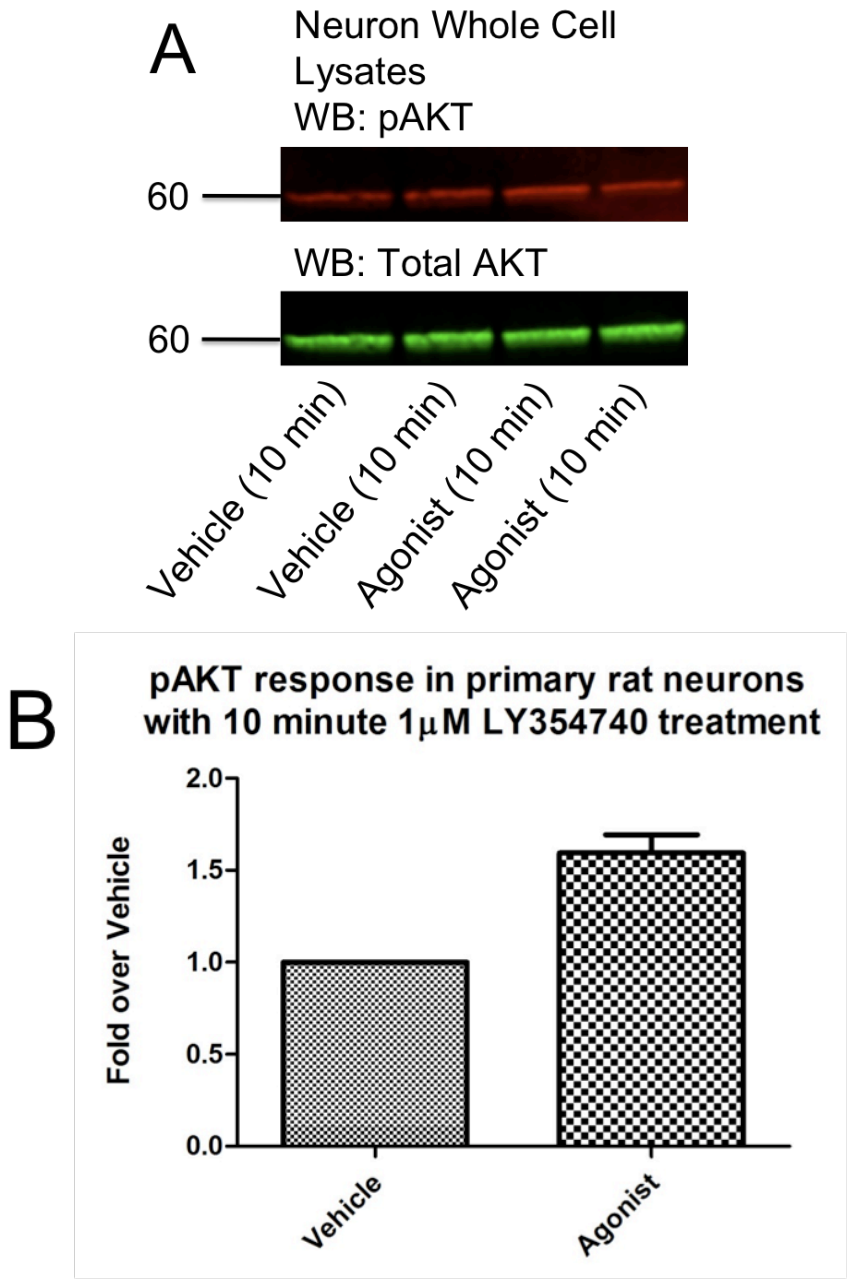

Figure 9: Group II mGluRs signal through pAKT in primary cortical embryonic rat neurons. A) A representative image with detection of phosphorylated AKT (red) and total AKT (green) following 10 minutes of treatment with vehicle or 1 μM agonist after 3 hours of starvation is shown. B) The pAKT to total AKT ratio increases 1.6-fold over vehicle after 10 minutes of agonist treatment (n=5; p=0.0038 in a paired t-test).

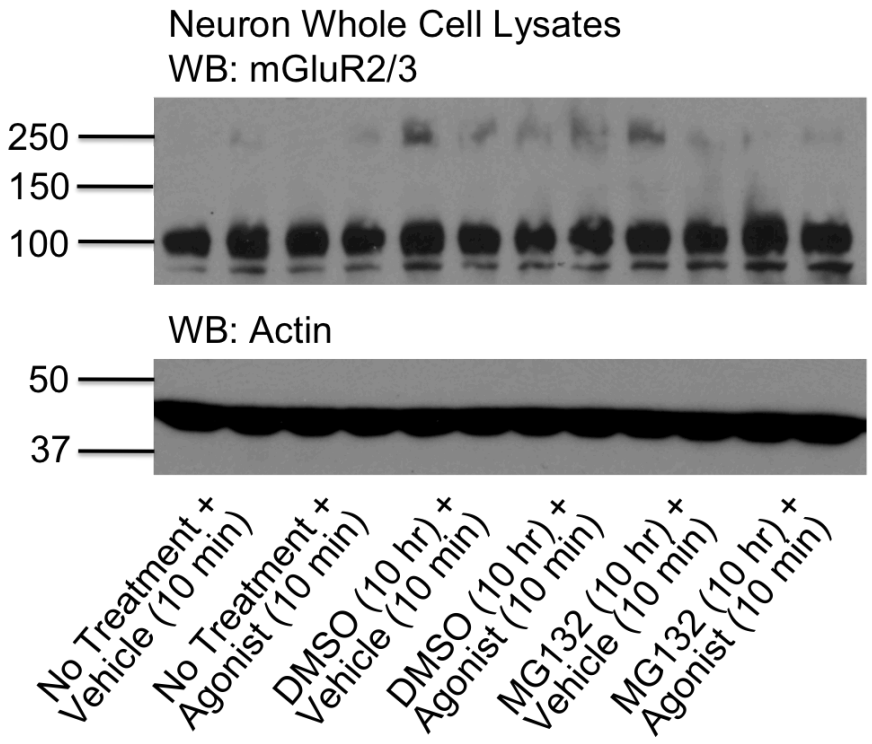

Figure 10: Effects of DMSO and MG132 on group II mGluRs are ablated when starvation occurs immediately before harvesting. A slight increase in the higher-order species may occur with DMSO treatment alone but not with MG132 dissolved in DMSO. The lack of a robust and consistent accumulation of group II mGluRs under conditions of DMSO and MG132 treatment followed by starvation precludes any valuable data collection from signaling assays that require such starvation.

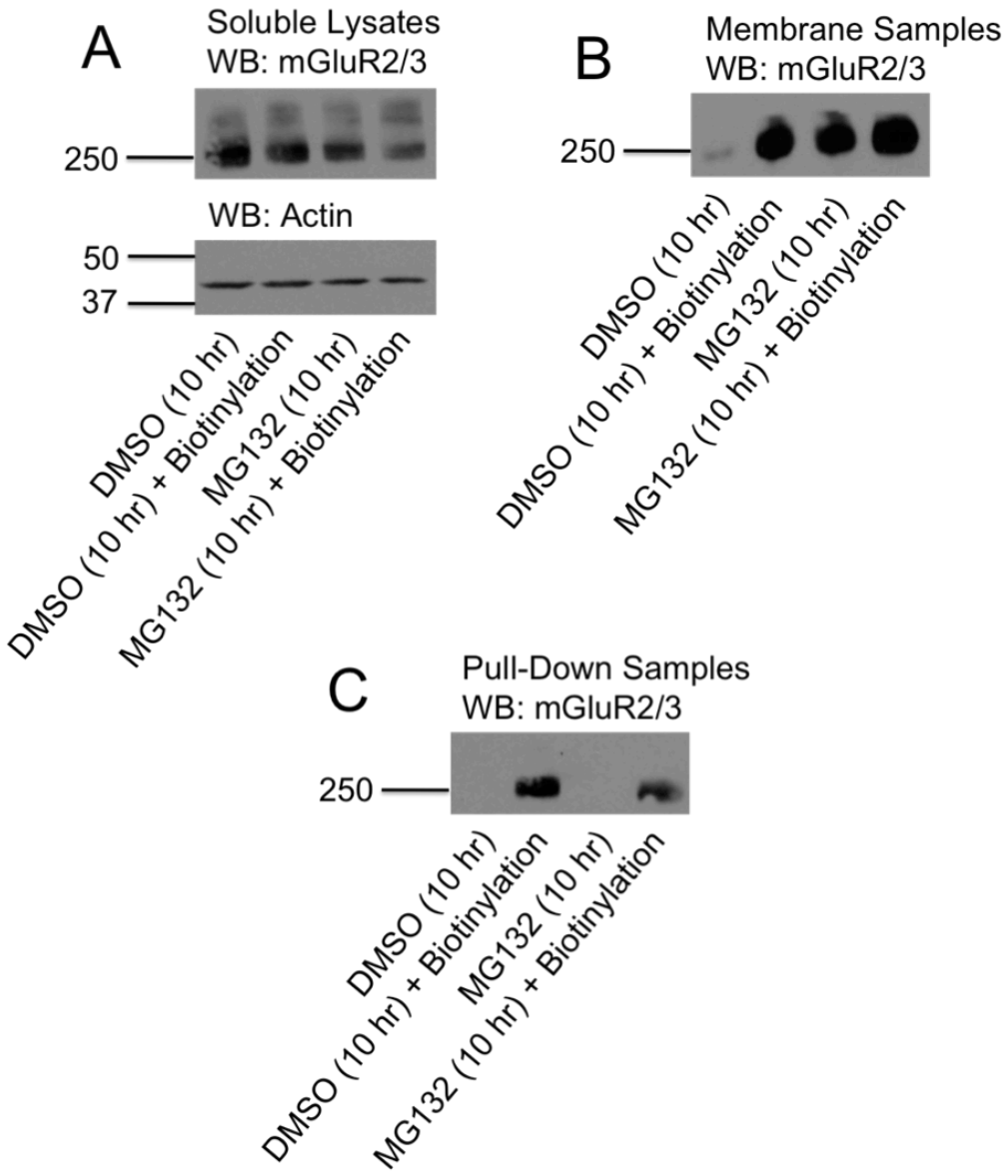

Figure 11: Effects of MG132 on mGluR3 surface expression cannot be evaluated with surface biotinylation due to solubility changes with MG132 treatment. A) No increase in mGluR3 detection with 10 μM MG132 treatment occurs in the soluble lysate samples, contrary to what has been seen in whole cell lysates. B) The biotinylation reaction and MG132 treatment both reduce mGluR3 solubility significantly, resulting in higher mGluR3 levels in the membrane fraction. C) Biotinylated surface mGluR3 from the 250 kDa higher-order species is pulled down with streptavidin beads. However, there is no difference in the amount of receptor pulled down between DMSO and MG132 conditions because the amount of receptor in the soluble lysates that are incubated with the beads is nearly equivalent.

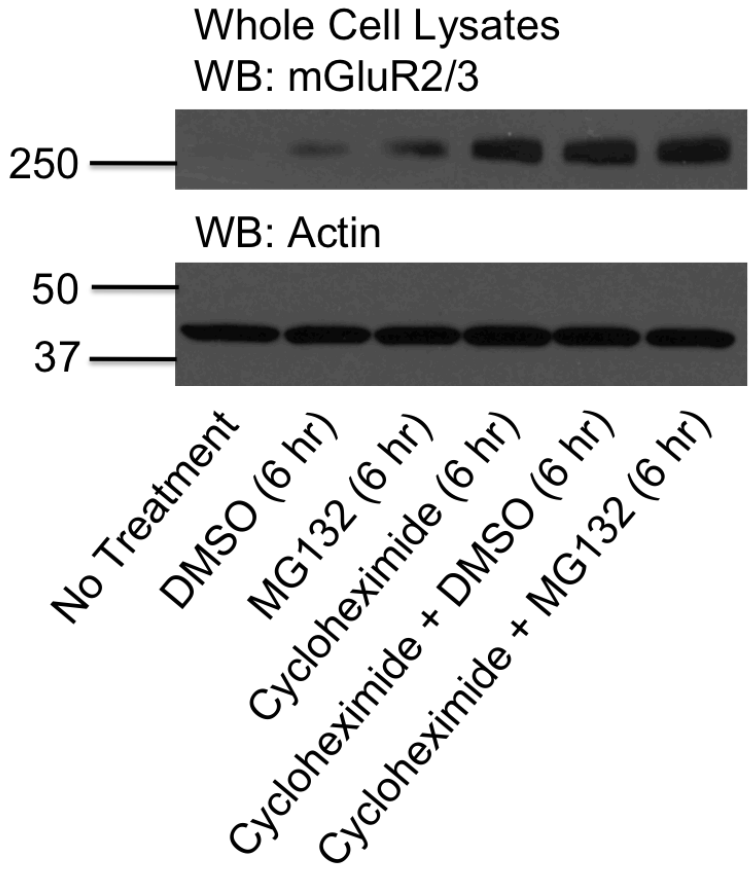

Figure 12: Cycloheximide appears to increase group II mGluR levels, contrary to known mechanism of action. In accordance with earlier studies, DMSO produces a large increase in mGluR2/3 expression over baseline, and MG132 has a moderate effect over DMSO. Cycloheximide should cause decreased protein expression by inhibiting protein biosynthesis, but unexpectedly, mGluR2/3 levels increased with cycloheximide application.

VII. References

Allison DB, Mentore JL, Heo M, Chandler LP, Cappelleri JC, et al. 1999. Antipsychotic-induced weight gain: a comprehensive research synthesis. *Am J Psychiatry* 156: 1686-96

Ambrosini A, Bresciani L, Fracchia S, Brunello N, Racagni G. 1995. Metabotropic glutamate receptors negatively coupled to adenylate cyclase inhibit N-methyl-D-aspartate receptor activity and prevent neurotoxicity in mesencephalic neurons in vitro. *Mol Pharmacol* 47: 1057-64

Andreasen NC, Olsen S. 1982. Negative v positive schizophrenia. Definition and validation. *Arch Gen Psychiatry* 39: 789-94

Arguello PA, Gogos JA. 2008. A signaling pathway AKTing up in schizophrenia. *J Clin Invest* 118: 2018-21

Association AP. 1994. *Diagnostic and statistical manual of mental disorders*. Washington, DC.

Baugh JM, Viktorova EG, Pilipenko EV. 2009. Proteasomes can degrade a significant proportion of cellular proteins independent of ubiquitination. *J Mol Biol* 386: 814-27

Baune BT, Suslow T, Beste C, Birosova E, Domschke K, et al. 2010. Association between genetic variants of the metabotropic glutamate receptor 3 (GRM3) and cognitive set shifting in healthy individuals. *Genes Brain Behav* 9: 459-66

Bergen SE, Petryshen TL. 2012. Genome-wide association studies of schizophrenia: does bigger lead to better results? *Curr Opin Psychiatry* 25: 76-82

Berger JV, Dumont AO, Focant MC, Vergouts M, Sternotte A, et al. 2012. Opposite regulation of metabotropic glutamate receptor 3 and metabotropic glutamate receptor 5 by inflammatory stimuli in cultured microglia and astrocytes. *Neuroscience* 205: 29-38

Bishop JR, Ellingrod VL, Moline J, Miller D. 2005. Association between the polymorphic GRM3 gene and negative symptom improvement during olanzapine treatment. *Schizophr Res* 77: 253-60

Blumcke I, Behle K, Malitschek B, Kuhn R, Knopfel T, et al. 1996. Immunohistochemical distribution of metabotropic glutamate receptor subtypes mGluR1b, mGluR2/3, mGluR4a and mGluR5 in human hippocampus. *Brain Res* 736: 217-26

Bohley P. 1995. The fates of proteins in cells. *Naturwissenschaften* 82: 544-50

Bostwick JR, Guthrie SK, Ellingrod VL. 2009. Antipsychotic-induced hyperprolactinemia. *Pharmacotherapy* 29: 64-73

Brown AS. 2011. Exposure to prenatal infection and risk of schizophrenia. *Front Psychiatry* 2: 63

Bruno V, Wroblewska B, Wroblewski JT, Fiore L, Nicoletti F. 1998. Neuroprotective activity of N-acetylaspartylglutamate in cultured cortical cells. *Neuroscience* 85: 751-7

Bulenger S, Marullo S, Bouvier M. 2005. Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol Sci* 26: 131-7

Cartmell J, Monn JA, Schoepp DD. 2000. Attenuation of specific PCP-evoked behaviors by the potent mGlu2/3 receptor agonist, LY379268 and comparison with the atypical antipsychotic, clozapine. *Psychopharmacology (Berl)* 148: 423-9

Chaki S, Yoshikawa R, Okuyama S. 2006. Group II metabotropic glutamate receptor-mediated regulation of dopamine release from slices of rat nucleus accumbens. *Neurosci Lett* 404: 182-6

Chavez-Noriega LE, Schaffhauser H, Campbell UC. 2002. Metabotropic glutamate receptors: potential drug targets for the treatment of schizophrenia. *Curr Drug Targets CNS Neurol Disord* 1: 261-81

Chavis P, Shinozaki H, Bockaert J, Fagni L. 1994. The metabotropic glutamate receptor types 2/3 inhibit L-type calcium channels via a pertussis toxin-sensitive G-protein in cultured cerebellar granule cells. *J Neurosci* 14: 7067-76

Chen Q, He G, Wu S, Xu Y, Feng G, et al. 2005. A case-control study of the relationship between the metabotropic glutamate receptor 3 gene and schizophrenia in the Chinese population. *Schizophr Res* 73: 21-6

Chiechio S, Copani A, Melchiorri D, Canudas AM, Storto M, et al. 2004. Metabotropic receptors as targets for drugs of potential use in the treatment of neuropathic pain. *J Endocrinol Invest* 27: 171-6

Conn PJ, Pin JP. 1997. Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol* 37: 205-37

Corti C, Crepaldi L, Mion S, Roth AL, Xuereb JH, Ferraguti F. 2007. Altered dimerization of metabotropic glutamate receptor 3 in schizophrenia. *Biol Psychiatry* 62: 747-55

D'Onofrio M, Cuomo L, Battaglia G, Ngomba RT, Storto M, et al. 2001. Neuroprotection

mediated by glial group-II metabotropic glutamate receptors requires the activation of the MAP kinase and the phosphatidylinositol-3-kinase pathways. *J Neurochem* 78: 435-45

David DC, Layfield R, Serpell L, Narain Y, Goedert M, Spillantini MG. 2002. Proteasomal degradation of tau protein. *J Neurochem* 83: 176-85

David HN, Ansseau M, Abraini JH. 2005. Dopamine-glutamate reciprocal modulation of release and motor responses in the rat caudate-putamen and nucleus accumbens of "intact" animals. *Brain Res Brain Res Rev* 50: 336-60

Dibonaventura MD, Gabriel S, Dupclay L, Gupta S, Kim E. 2012. A patient perspective of the impact of medication side effects on adherence: results of a cross-sectional nationwide survey of patients with schizophrenia. *BMC Psychiatry* 12: 20

Durand D, Carniglia L, Caruso C, Lasaga M. 2011. Reduced cAMP, Akt activation and p65-c-Rel dimerization: mechanisms involved in the protective effects of mGluR3 agonists in cultured astrocytes. *PLoS One* 6: e22235

Egan MF, Straub RE, Goldberg TE, Yakub I, Callicott JH, et al. 2004. Variation in GRM3 affects cognition, prefrontal glutamate, and risk for schizophrenia. *Proc Natl Acad Sci U S A* 101: 12604-9

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA. 2004. Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat Genet* 36: 131-7

Foord SM, Bonner TI, Neubig RR, Rosser EM, Pin JP, et al. 2005. International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacol Rev* 57: 279-88

Freedman R. 2003. Schizophrenia. *N Engl J Med* 349: 1738-49

Freyberg Z, Ferrando SJ, Javitch JA. 2010. Roles of the Akt/GSK-3 and Wnt signaling pathways in schizophrenia and antipsychotic drug action. *Am J Psychiatry* 167: 388-96

Gejman PV, Sanders AR, Duan J. 2010. The role of genetics in the etiology of schizophrenia. *Psychiatr Clin North Am* 33: 35-66

Gejman PV, Sanders AR, Kendler KS. 2011. Genetics of schizophrenia: new findings and challenges. *Annu Rev Genomics Hum Genet* 12: 121-44

Ghose S, Gleason KA, Potts BW, Lewis-Amezcua K, Tamminga CA. 2009. Differential expression of metabotropic glutamate receptors 2 and 3 in schizophrenia: a mechanism for antipsychotic drug action? *Am J Psychiatry* 166: 812-20

Giordano F, Simoes S, Raposo G. 2011. The ocular albinism type 1 (OA1) GPCR is ubiquitinated and its traffic requires endosomal sorting complex responsible for transport (ESCRT) function. *Proc Natl Acad Sci U S A* 108: 11906-11

Greenslade RG, Mitchell SN. 2004. Selective action of (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268), a group II metabotropic glutamate receptor agonist, on basal and phencyclidine-induced dopamine release in the nucleus accumbens shell. *Neuropharmacology* 47: 1-8

Gruber CW, Muttenthaler M, Freissmuth M. 2010. Ligand-based peptide design and combinatorial peptide libraries to target G protein-coupled receptors. *Curr Pharm Des* 16: 3071-88

Hamm HE. 1998. The many faces of G protein signaling. *J Biol Chem* 273: 669-72

Hanslick JL, Lau K, Noguchi KK, Olney JW, Zorumski CF, et al. 2009. Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system. *Neurobiol Dis* 34: 1-10

Harrison PJ, Lyon L, Sartorius LJ, Burnet PW, Lane TA. 2008. The group II metabotropic glutamate receptor 3 (mGluR3, mGlu3, GRM3): expression, function and involvement in schizophrenia. *J Psychopharmacol* 22: 308-22

Hepler JR, Gilman AG. 1992. G proteins. *Trends Biochem Sci* 17: 383-7

Hershko A, Ciechanover A. 1998. The ubiquitin system. *Annu Rev Biochem* 67: 425-79

Hirsch SR, Das I, Garey LJ, de Belleroche J. 1997. A pivotal role for glutamate in the pathogenesis of schizophrenia, and its cognitive dysfunction. *Pharmacol Biochem Behav* 56: 797-802

Iacovelli L, Molinaro G, Battaglia G, Motolese M, Di Menna L, et al. 2009. Regulation of group II metabotropic glutamate receptors by G protein-coupled receptor kinases: mGlu2 receptors are resistant to homologous desensitization. *Mol Pharmacol* 75: 991-1003

Jariel-Encontre I, Bossis G, Piechaczyk M. 2008. Ubiquitin-independent degradation of proteins by the proteasome. *Biochim Biophys Acta* 1786: 153-77

Kane JM. 1996. Treatment-resistant schizophrenic patients. *J Clin Psychiatry* 57 Suppl 9: 35-40

Kegeles LS, Abi-Dargham A, Zea-Ponce Y, Rodenhiser-Hill J, Mann JJ, et al. 2000. Modulation of amphetamine-induced striatal dopamine release by ketamine in humans: implications for schizophrenia. *Biol Psychiatry* 48: 627-40

Kim JS, Kornhuber HH, Schmid-Burgk W, Holzmuller B. 1980. Low cerebrospinal fluid glutamate in schizophrenic patients and a new hypothesis on schizophrenia. *Neurosci Lett* 20: 379-82

Kokkinidis L, Anisman H. 1981. Amphetamine psychosis and schizophrenia: a dual model. *Neurosci Biobehav Rev* 5: 449-61

Kontoyianni M, Liu Z. 2012. Structure-based design in the GPCR target space. *Curr Med Chem* 19: 544-56

Krabbendam L, van Os J. 2005. Schizophrenia and urbanicity: a major environmental influence--conditional on genetic risk. *Schizophr Bull* 31: 795-9

Krupnick JG, Santini F, Gagnon AW, Keen JH, Benovic JL. 1997. Modulation of the arrestin-clathrin interaction in cells. Characterization of beta-arrestin dominant-negative mutants. *J Biol Chem* 272: 32507-12

Laruelle M, Kegeles LS, Abi-Dargham A. 2003. Glutamate, dopamine, and schizophrenia: from pathophysiology to treatment. *Ann N Y Acad Sci* 1003: 138-58

Lee KW, Woon PS, Teo YY, Sim K. 2012. Genome wide association studies (GWAS) and copy number variation (CNV) studies of the major psychoses: what have we learnt? *Neurosci Biobehav Rev* 36: 556-71

Linden AM, Shannon H, Baez M, Yu JL, Koester A, Schoepp DD. 2005. Anxiolytic-like activity of the mGLU2/3 receptor agonist LY354740 in the elevated plus maze test is disrupted in metabotropic glutamate receptor 2 and 3 knock-out mice. *Psychopharmacology (Berl)* 179: 284-91

Luttrell LM, Lefkowitz RJ. 2002. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 115: 455-65

Lyon L, Burnet PW, Kew JN, Corti C, Rawlins JN, et al. 2011. Fractionation of spatial memory in GRM2/3 (mGlu2/mGlu3) double knockout mice reveals a role for group II metabotropic glutamate receptors at the interface between arousal and cognition. *Neuropsychopharmacology* 36: 2616-28

Makoff A, Volpe F, Lelchuk R, Harrington K, Emson P. 1996. Molecular characterization and localization of human metabotropic glutamate receptor type 3. *Brain Res Mol Brain Res* 40: 55-63

Malhotra AK, Pinals DA, Weingartner H, Sirocco K, Missar CD, et al. 1996. NMDA receptor function and human cognition: the effects of ketamine in healthy volunteers. *Neuropsychopharmacology* 14: 301-7

Margolese HC, Chouinard G, Kolivakis TT, Beauclair L, Miller R. 2005. Tardive dyskinesia in the era of typical and atypical antipsychotics. Part 1: pathophysiology and mechanisms of induction. *Can J Psychiatry* 50: 541-7

McEntee WJ, Crook TH. 1993. Glutamate: its role in learning, memory, and the aging brain. *Psychopharmacology (Berl)* 111: 391-401

Melo SP, Barbour KW, Berger FG. 2011. Cooperation between an intrinsically disordered region and a helical segment is required for ubiquitin-independent degradation by the proteasome. *J Biol Chem* 286: 36559-67

Meltzer HY, Li Z, Kaneda Y, Ichikawa J. 2003. Serotonin receptors: their key role in drugs to treat schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry* 27: 1159-72

Milligan G. 2004. G protein-coupled receptor dimerization: function and ligand pharmacology. *Mol Pharmacol* 66: 1-7

Minneman KP. 2007. Heterodimerization and surface localization of G protein coupled receptors. *Biochem Pharmacol* 73: 1043-50

Mizushima N. 2007. Autophagy: process and function. *Genes Dev* 21: 2861-73

Moghaddam B, Adams BW. 1998. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. *Science* 281: 1349-52

Moore TH, Zammit S, Lingford-Hughes A, Barnes TR, Jones PB, et al. 2007. Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. *Lancet* 370: 319-28

Moreno JL, Sealfon SC, Gonzalez-Maeso J. 2009. Group II metabotropic glutamate receptors and schizophrenia. *Cell Mol Life Sci* 66: 3777-85

Moriyoshi K, Iijima K, Fujii H, Ito H, Cho Y, Nakanishi S. 2004. Seven in absentia homolog 1A mediates ubiquitination and degradation of group 1 metabotropic glutamate receptors. *Proc Natl Acad Sci U S A* 101: 8614-9

Muench J, Hamer AM. 2010. Adverse effects of antipsychotic medications. *Am Fam Physician* 81: 617-22

Nakanishi S. 1992. Molecular diversity of glutamate receptors and implications for brain function. *Science* 258: 597-603

Nakanishi S, Masu M, Bessho Y, Nakajima Y, Hayashi Y, Shigemoto R. 1994. Molecular diversity of glutamate receptors and their physiological functions. *Exs* 71: 71-80

Neves SR, Ram PT, Iyengar R. 2002. G protein pathways. *Science* 296: 1636-9

Newcomer JW, Farber NB, Jevtovic-Todorovic V, Selke G, Melson AK, et al. 1999. Ketamine-induced NMDA receptor hypofunction as a model of memory impairment and psychosis. *Neuropsychopharmacology* 20: 106-18

Norton N, Williams HJ, Dwyer S, Ivanov D, Preece AC, et al. 2005. No evidence for association between polymorphisms in GRM3 and schizophrenia. *BMC Psychiatry* 5: 23

Ohishi H, Shigemoto R, Nakanishi S, Mizuno N. 1993. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR3) in the rat brain: an in situ hybridization study. *J Comp Neurol* 335: 252-66

Ohnuma T, Augood SJ, Arai H, McKenna PJ, Emson PC. 1998. Expression of the human excitatory amino acid transporter 2 and metabotropic glutamate receptors 3 and 5 in the prefrontal cortex from normal individuals and patients with schizophrenia. *Brain Res Mol Brain Res* 56: 207-17

Olney JW, Farber NB. 1995. NMDA antagonists as neurotherapeutic drugs, psychotogens, neurotoxins, and research tools for studying schizophrenia. *Neuropsychopharmacology* 13: 335-45

Olney JW, Newcomer JW, Farber NB. 1999. NMDA receptor hypofunction model of schizophrenia. *J Psychiatr Res* 33: 523-33

Patil ST, Zhang L, Martenyi F, Lowe SL, Jackson KA, et al. 2007. Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nat Med* 13: 1102-7

Paz RD, Ortiz T, Canive JM. 2007. [Auditory gating deficits in schizophrenia: unimodal or heteromodal dysfunction?]. *Actas Esp Psiquiatr* 35: 208-18

Petralia RS, Wang YX, Niedzielski AS, Wenthold RJ. 1996. The metabotropic glutamate receptors, mGluR2 and mGluR3, show unique postsynaptic, presynaptic and glial localizations. *Neuroscience* 71: 949-76

Pieters T, Majerus B. 2011. The introduction of chlorpromazine in Belgium and the Netherlands (1951-1968); tango between old and new treatment features. *Stud Hist Philos Biol Biomed Sci* 42: 443-52

Pizzi M, Consolandi O, Memo M, Spano PF. 1996. Activation of multiple metabotropic glutamate receptor subtypes prevents NMDA-induced excitotoxicity in rat hippocampal slices. *Eur J Neurosci* 8: 1516-21

Quintero J, Barbudo del Cura E, Lopez-Ibor MI, Lopez-Ibor JJ. 2011. The evolving concept of Treatment- Resistant Schizophrenia. *Actas Esp Psiquiatr* 39: 236-50

Ray WA, Chung CP, Murray KT, Hall K, Stein CM. 2009. Atypical antipsychotic drugs and the risk of sudden cardiac death. *N Engl J Med* 360: 225-35

Saur SJ, Sangkhae V, Geddis AE, Kaushansky K, Hitchcock IS. 2010. Ubiquitination and degradation of the thrombopoietin receptor c-Mpl. *Blood* 115: 1254-63

Seeman P. 1987. Dopamine receptors and the dopamine hypothesis of schizophrenia. *Synapse* 1: 133-52

Sherman AD, Davidson AT, Baruah S, Hegwood TS, Waziri R. 1991. Evidence of glutamatergic deficiency in schizophrenia. *Neurosci Lett* 121: 77-80

Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, et al. 1997. Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J Neurosci* 17: 7503-22

Simon MI, Strathmann MP, Gautam N. 1991. Diversity of G proteins in signal transduction. *Science* 252: 802-8

Sorensen HJ, Mortensen EL, Reinisch JM, Mednick SA. 2009. Association between prenatal exposure to bacterial infection and risk of schizophrenia. *Schizophr Bull* 35: 631-7

Stone JM, Pilowsky LS. 2007. Novel targets for drugs in schizophrenia. *CNS Neurol Disord Drug Targets* 6: 265-72

Sullivan PF, Kendler KS, Neale MC. 2003. Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Arch Gen Psychiatry* 60: 1187-92

Sutton LP, Rushlow WJ. 2011. Regulation of Akt and Wnt signaling by the group II metabotropic glutamate receptor antagonist LY341495 and agonist LY379268. *J Neurochem* 117: 973-83

Tanabe Y, Nomura A, Masu M, Shigemoto R, Mizuno N, Nakanishi S. 1993. Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J Neurosci* 13: 1372-8

Tandon R. 1999. Cholinergic aspects of schizophrenia. *Br J Psychiatry Suppl*: 7-11

Tandon R. 2011. Antipsychotics in the treatment of schizophrenia: an overview. *J Clin Psychiatry* 72 Suppl 1: 4-8

Tatarczynska E, Klodzinska A, Kroczka B, Chojnacka-Wojcik E, Pilc A. 2001. The

antianxiety-like effects of antagonists of group I and agonists of group II and III metabotropic glutamate receptors after intrahippocampal administration. *Psychopharmacology (Berl)* 158: 94-9

Tenback DE, van Harten PN. 2011. Epidemiology and risk factors for (tardive) dyskinesia. *Int Rev Neurobiol* 98: 211-30

Tochigi M, Suga M, Ohashi J, Otowa T, Yamasue H, et al. 2006. No association between the metabotropic glutamate receptor type 3 gene (GRM3) and schizophrenia in a Japanese population. *Schizophr Res* 88: 260-4

Tsai G, Passani LA, Slusher BS, Carter R, Baer L, et al. 1995. Abnormal excitatory neurotransmitter metabolism in schizophrenic brains. *Arch Gen Psychiatry* 52: 829-36

van Os J, Kapur S. 2009. Schizophrenia. *Lancet* 374: 635-45

Vondracek J, Soucek K, Sheard MA, Chramostova K, Andrysik Z, et al. 2006. Dimethyl sulfoxide potentiates death receptor-mediated apoptosis in the human myeloid leukemia U937 cell line through enhancement of mitochondrial membrane depolarization. *Leuk Res* 30: 81-9

Wettschureck N, Offermanns S. 2005. Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85: 1159-204

Wu EQ, Birnbaum HG, Shi L, Ball DE, Kessler RC, et al. 2005. The economic burden of schizophrenia in the United States in 2002. *J Clin Psychiatry* 66: 1122-9

Yokoi M, Kobayashi K, Manabe T, Takahashi T, Sakaguchi I, et al. 1996. Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. *Science* 273: 645-7

Zetter BR, Mangold U. 2005. Ubiquitin-independent degradation and its implication in cancer. *Future Oncol* 1: 567-70