IDENTIFYING FUNCTIONALLY SELECTIVE LIGANDS FOR THE SEROTONIN-2A RECEPTOR

By

Amanda Ladd

Submitted to the graduate degree program in Pharmacology and Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

_____

Chairperson: Nancy A. Muma, Ph.D.

_____

Honglian Shi, Ph.D.

_____

Liqin Zhao, Ph.D.

Date Defended: January 26, 2017

The Thesis Committee for Amanda Ladd

certifies that this is the approved version of the following thesis:

IDENTIFYING FUNCTIONALLY SELECTIVE LIGANDS FOR THE SEROTONIN-2A RECEPTOR

_____

Chairperson: Nancy A. Muma, Ph.D.

Date approved: January 27, 2017

Abstract

Atypical antipsychotics are used to treat schizophrenia and although atypical antipsychotics helped improve the management of positive symptoms, many patients are still suffering from negative and cognitive symptoms and side effects. Serotonin-2A receptors have been implicated in the mechanism of action of atypical antipsychotics. Atypical antipsychotics such as olanzapine and clozapine are antagonists of 5-HT2A/$G\alpha_{q/11}$ pathway, but activate the 5-HT2A/JAK2/STAT3 pathway. Studying the functional selectivity of atypical antipsychotics at the 5-HT2A receptor may lead to a better understanding of the role that 5-HT2A receptors play in the therapeutic effect of atypical antipsychotics. This dissertation investigates five analogs of olanzapine and the 5-HT2A receptor antagonist, ketanserin, for functional selectivity at the 5-HT2A receptor. A calcium mobilization assay was used to investigate the 5-HT2A/$G\alpha_{q/11}$ pathway and immunoblotting was used to investigate the 5-HT2A/JAK2/STAT3. We identified three analogs that induced calcium mobilization and two analogs that prevented 5-HT2A receptor agonist induced calcium mobilization. We also found that four analogs induced phosphorylation of JAK2 and one analog that prevented 5-HT2A receptor agonist induced phosphorylation of JAK2. In the future, functionally selective analogs can be used to study the 5-HT2A/$G\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 pathways separately in order to gain a better understanding of the role each individual pathway plays in the therapeutic effect of atypical antipsychotics.

Acknowledgements

First, I would like to thank my mentor, Nancy Muma, for her guidance and patience throughout this entire process. Because of her, I have become a better scientist and learned so much more than I can express in words. Thank you to my committee members, Honglian Shi and Liqin Zhao, for their helpful suggestions for my experiments. Also, without the analogs created by Dr. Prisinzano's laboratory, this dissertation would not be possible.

To my wonderful labmates: Dr. Zhen Mi, you taught me everything from cell culture to western blotting and I learned so much under your tutelage. Mengya Wang, you are such a great friend and I enjoyed our work together. You were always willing to help me if I needed anything. To Yusheng Li, I could always count on you to brighten my day and cheer me on. And to Khushboo Kapadia, you have been the best friend a person could ask for. You supported me both in the lab and outside of it. To the rest of my Kansas family, thank you to everyone who supported me during the past two years. I will never forget your kindness and friendship.

None of this would be possible without my Texas family. I love you all. Every phone call and trip north meant the world to me. My parents have always supported me, even when it came to moving to another state. Their encouragement and love is the reason I'm writing this dissertation. Thank you.

Table of Contents

List of Figures and Tables

List of Abbreviations

5-HT	serotonin
AA	arachidonic acid
Akt	protein kinase B
AUC	area under the curve
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREB	cAMP response element-binding protein
DAG	diacylglycerol
DOI	2,5-dimethoxy-4-iodoamphetamine HCl
ECL	enhanced chemiluminescence
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FBS	fetal bovine serum
GPCR	G protein coupled receptor
HRP	horseradish peroxidase
IgG	immunoglobulin G
IOD	integrated optical density
IP3	inositol triphosphate

JAK	janus kinase
LSD	lysergic acid diethylamide
MAPK	mitogen-activated protein kinases
MEK	mitogen-activated protein kinase kinase
NMDA	N-methyl-D-aspartate
OCD	obsessive compulsive disorder
PBS	phosphate buffered saline
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
Rac1	Ras-related C3 botulinum toxin substrate
RhoA	Ras-homolog A
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT	signal transducer and activator of transcription
TBST	Tris-buffered saline with Tween
VSM	vascular smooth muscle

Chapter 1: Introduction

Schizophrenia

Schizophrenia is a debilitating psychological disorder that affects approximately 1% of the population. Symptoms of schizophrenia can be divided into positive, negative, and cognitive symptoms. Positive symptoms include delusions, hallucinations, and an overall loss of reality. Negative symptoms include dramatic changes to personality, impaired motivation, social isolation, emotional dysregulation, and disorganized behavior. Cognitive symptoms include attention and memory deficits as well as disturbances in thought processes (Joyce and Roiser 2007, Owen, Sawa et al. 2016). The onset of symptoms usually occurs between the ages of 16-25, but because these symptoms vary between patients, schizophrenia is often difficult to diagnose. In addition, schizophrenia is a chronic disorder and the manifestation of symptoms can change throughout a patient's lifespan (Lieberman, Perkins et al. 2001, Addington and Heinssen 2012).

Currently, the cause for schizophrenia is unknown, but it is hypothesized that schizophrenia could be due to a combination of molecular, cellular, and/or structural abnormalities in the brain (NIMH 2016). Although there is an increased risk of schizophrenia when family members also have the disorder, there are currently no genetic biomarkers that can definitively identify people who will develop schizophrenia (Fullard, Halene et al. 2016). Environmental factors such as stresses during early neurodevelopment, socioeconomic factors, childhood trauma, brain injuries, infections, drug use and other disorders (such as epilepsy) have correlated to an increased risk of schizophrenia (Kunugi, Urushibara et al. 2003, Khandaker, Zimbron et al. 2013, Owen, Sawa et al. 2016).

There are studies to support that irregularities in neurotransmission may play a role in the manifestation of symptoms. For example, dysfunction of dopaminergic signaling is implicated in psychotic symptoms (Creese, Burt et al. 1976, Abi-Dargham 2014). There are also theories that glutamatergic dysfunction could contribute to cognitive symptoms of schizophrenia (Moghaddam and Javitt 2012). Alterations of N-methyl-D-aspartate (NMDA) glutamate receptors can lead to impaired synchronization of neuronal firing, thus leading to impaired cognitive function (Kantrowitz and Javitt 2010). Irregularities of serotonergic signaling may also contribute to altered function seen in schizophrenia (Meltzer, Li et al. 2003, Aznar and Hervig Mel 2016).

Cellular stress is theorized to contribute to the pathology of schizophrenia. Stress associated signaling pathways, such as those related to inflammatory responses and oxidative stress, normally regulate the development and maintenance of synaptic connectivity, but in schizophrenic patients there are abnormalities in interneurons and dendritic spines (Schafer, Lehrman et al. 2012, Ji, Miyauchi et al. 2013). There is evidence that oxidative stress may be causing deficits in myelination needed for signal transduction (Cabungcal, Counotte et al. 2014, Sawa and Seidman 2014). Altered inflammatory responses could be contributing to dysfunction of astrocytes and microglia that are known to maintain dendritic spines (Owen, Sawa et al. 2016).

Antipsychotics

There is currently no cure for schizophrenia and management of symptoms depends on antipsychotics and psychotherapy. First generation antipsychotics, also known as typical antipsychotics, were developed in the 1950s after it was discovered that chlorpromazine could reduce the psychotic symptoms of schizophrenic patients through antagonism of dopamine D2

receptors (Shen 1999, Seeman 2010). More potent typical antipsychotics, such as haloperidol, loxapine, and perphenazine, were developed in the 1960s and 1970s to antagonize dopamine D2 receptors. The first generation antipsychotics were effective in managing positive symptoms such as delusions and hallucinations in some patients, but were accompanied by severe extrapyramidal side effects such as Parkinsonism, akathisia, acute dystonia, and tardive dyskinesia (Barnes and McPhillips 1998, Llorca, Chereau et al. 2002). Due to the debilitating side effects and ineffective treatment of negative and cognitive symptoms, the second generation antipsychotics were created.

Second generation antipsychotics, also known as atypical antipsychotics, are also antagonists of dopamine D2 receptors, but this antagonism is no longer the only therapeutic target. Atypical antipsychotics also antagonize dopamine D1 and D4 receptors as well as histaminergic, muscarinic, and serotoninergic receptors (Bymaster, Calligaro et al. 1996, Arnt 1998). Because of these diverse interactions, atypical antipsychotics were more effective in improving positive symptoms and patients also reported improvements of negative and cognitive symptoms. The extrapyramidal side effects of atypical antipsychotics were greatly reduced compared to typical antipsychotics, however atypical antipsychotics have other side effects such as weight gain, metabolic syndrome, diabetes, sexual dysfunction, and cardiovascular disease (Ucok and Gaebel 2008). Researchers have hypothesized that the improved management of negative and cognitive symptoms could be due to interaction with serotonin receptors (Meltzer 1999, Amato 2015).

Atypical antipsychotics, such as olanzapine and clozapine, antagonize serotonin receptors, such as serotonin-2A and 2C receptors. Atypical antipsychotics can lead to desensitization and down-regulation of serotonin-2A (5-HT2A) receptors and it has been

suggested that this effect on 5-HT2A receptors could be contributing to the antipsychotic effects (Meltzer, Li et al. 2003, Amato 2015).

Serotonin Receptors

The neurotransmitter serotonin (5-HT) and serotonin receptors modulate a number of behavioral processes such as mood, perception, aggression, memory, sexuality, and attention (Berger, Gray et al. 2009). There are seven families of serotonin receptors, 5-HT1-5HT7 receptors. The family of 5-HT1 receptors contain five receptors: 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F (Hoyer, Clarke et al. 1994). 5-HT1 receptors are G-protein coupled receptors (GPCRs) that negatively regulate adenylyl cyclase and cAMP (Polter and Li 2010). The 5-HT3 receptor is the only 5-HT receptor that is an ionotropic receptor and activation of the receptor causes depolarization through the opening of cation channels (Costall and Naylor 2004). 5-HT4, 5-HT6, and 5-HT7 are GPCRs that couple to Gαs proteins that positively regulate adenylyl cyclase and cAMP (Berger, Gray et al. 2009). The 5-HT5 receptor has not been thoroughly characterized, but it is thought to be similar to 5-HT1 receptors (Hoyer, Clarke et al. 1994).

5-HT2A Receptors

In the family of 5-HT2 receptors, there are three receptors: 5-HT2A, 5-HT2B, and 5-HT2C (Eison and Mullins 1996). The three subtypes have a 45-50% sequence identity and the transmembrane domains of 5-HT2A and 5-HT2C receptors share 80% of their sequence (Blaazer, Smid et al. 2008). 5-HT2A receptors are pleiotropic GPCRs expressed within regions of the brain including the cortex, the claustrum, entorhinal and piriform cortices, the basal ganglia, and the hippocampus (Barnes and Sharp 1999). The receptor is also found in blood platelets, smooth muscle, and skeletal muscle (Cook, Fletcher et al. 1994, Fiorica-Howells, Hen et al. 2002). 5-HT2A receptors are located on dendrites of pyramidal glutamatergic neurons,

GABAergic interneurons, and cholinergic neurons (Blaazer, Smid et al. 2008). 5-HT2A receptors in the central nervous system (CNS) can modulate GABAergic and glutamatergic neurotransmission, the secretion of hormones, and neuronal plasticity (Vaidya, Marek et al. 1997, Van de Kar, Javed et al. 2001, Leysen 2004). In the periphery, vasoconstriction and platelet aggregation are regulated by 5-HT2A receptors (Leysen, de Chaffoy de Courcelles et al. 1984).

The 5-HT2A receptor has been implicated in schizophrenia, depression, anxiety, obsessive compulsive disorders (OCD), and attention deficit hyperactivity disorder. In postmortem studies of depressed patients and patients who have committed suicide, 5-HT2A receptors were increased in the brain (Dwivedi, Mondal et al. 2005). Likewise, patients with OCD show increased 5-HT2A receptor binding (Adams, Hansen et al. 2005). In the postmortem studies of schizophrenic patients, 5-HT2A receptors are decreased in the cortex (Bennett, Enna et al. 1979, Matsumoto, Inoue et al. 2005). Therefore, the receptor is a target for antipsychotics, antidepressants, and other therapeutic drugs. In animal studies, antidepressants have been shown to decrease 5-HT2A receptors in the brain, suggesting a role of 5-HT2A in the action of antidepressants. As stated before, atypical antipsychotics bind to the 5-HT2A receptor, as well as hallucinogens that produce schizophrenic-like symptoms, indicating a relationship between 5-HT2A receptors and schizophrenia (Muschamp, Regina et al. 2004).

Signaling Pathways of 5-HT2A Receptors

The 5-HT2A receptors are GPCRs that have seven-transmembrane structures that couple to G-proteins. Like the other members of the 5-HT2 subfamily, the 5-HT2A receptor couples with $G\alpha_{q/11}$ proteins to activate phospholipase C (PLC). Activation of PLC catalyzes the formation of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to the IP3 receptor

on the endoplasmic reticulum and leads to the release of calcium into the cytosol. DAG activates the protein kinase C (PKC) cascade (Barnes and Sharp 1999, Raote, Bhattacharya et al. 2007). Through activation of the 5-HT2A/$G\alpha_{q/11}$ pathway, transamidation of small G proteins such as Rac1, RhoA, and Rab4 can occur (Walther, Peter et al. 2003, Dai, Han et al. 2008, Muma and Mi 2015).

Separate from the $G\alpha_{q/11}$ pathway, 5-HT2A activates phospholipase A2 (PLA2) and arachidonic acid (AA) (Berg, Clarke et al. 1994, Berg, Maayani et al. 1998). Activation of the PLA2/AA pathway can occur through 5-HT2A/$G\alpha_{i/o}$ mediated Ras-Raf-MEK-ERK signaling or through 5-HT2A/ $G\alpha_{12/13}$ mediated RhoA-MAPK phosphorylation of PLA2 (Kurrasch-Orbaugh, Parrish et al. 2003, Kurrasch-Orbaugh, Watts et al. 2003). 5-HT2A receptors have also been linked to changes in nitric oxide, calmodulin, CREB, and Akt (Miller, Mariano et al. 1997, Van de Kar, Javed et al. 2001).

In skeletal muscle, vascular smooth muscle (VSM), and neuronal cells, the 5-HT2A receptor activates janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathways (Guillet-Deniau, Burnol et al. 1997, Banes, Shaw et al. 2005, Singh, Shi et al. 2007). There are four members of the JAK family (JAK1, JAK2, JAK3, and TYK2) and seven members of the STAT family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) (Aaronson and Horvath 2002). JAK/STAT signaling pathways can be activated through cytokines such as interferon and interleukin or GPCRs. JAKs recruited by the receptor autophosphorylate and bind to SH2 domains of STATs. The STATs are tyrosine-phosphorylated by the JAK and form dimers. The STAT dimers translocate to the nucleus and regulate the transcription of certain genes. 5-HT2A receptors have been reported to interact with JAK1, JAK2, STAT1, and STAT3 depending on the location of the receptors. In skeletal muscle,

serotonin stimulates JAK2/STAT3 via the 5-HT2A receptor; in VSM, 5-HT2A interacts with JAK1, JAK2, and STAT1; and in neurons the 5-HT2A receptor stimulates JAK2/STAT3 (Guillet-Deniau, Burnol et al. 1997, Banes, Shaw et al. 2005, Singh, Shi et al. 2007).

Functional Selectivity of 5-HT2A Receptors

Functional selectivity describes the ligand-dependent selectivity for different signaling pathways for a receptor (Kenakin 1995, Kenakin 2001). There are several receptors such as the u-opioid receptor, β2-adrenergic, V2 vasopressin, and dopamine receptors that show functional selectivity (He, Fong et al. 2002, Urban, Clarke et al. 2007). The 5-HT2A receptor was first investigated as functionally selective after it was discovered that the hallucinogenic effects of lysergic acid diethylamide (LSD), which binds the 5-HT2A receptor, did not correlate to the known effects of 5-HT2A/$G\alpha_{q/11}$ signaling (Muschamp, Regina et al. 2004, Urban, Clarke et al. 2007). The functional selectivity of 5-HT2A receptors plays a role in receptor regulation, internalization, recycling, and dimerization and can depend on ligand, G-proteins, cell type, receptor expression, and structural conformation. 5-HT2A receptors independently activate the 5-HT2A/$G\alpha_{q/11}$ and the 5-HT2A/PLA2 pathways depending on the ligand structure (Kurrasch-Orbaugh, Watts et al. 2003, Moya, Berg et al. 2007). Different ligands such as serotonin, dopamine, DOI, and clozapine cause internalization of the 5-HT2A receptor at different rates (Raote, Bhattacharyya et al. 2013). Depending on cell type, the 5-HT2A receptor selectively activates different isoforms of JAK/STATs (Guillet-Deniau, Burnol et al. 1997, Banes, Shaw et al. 2005, Singh, Shi et al. 2007).

Statement of Purpose

Schizophrenia affects approximately 1% of the population and there is no cure for the disorder. Although the use of atypical antipsychotics has improved the management of positive

symptoms for many patients without the detrimental extrapyramidal side effects and has slightly improved the management of negative and cognitive symptoms, there are still many schizophrenic patients that are suffering from negative and cognitive symptoms (Buckley and Stahl 2007, Goldberg, Goldman et al. 2007). For some patients, the risk of the side effects deters them from using antipsychotics. Therefore, there is a demand for improved antipsychotics that address the negative and cognitive symptoms, as well as reduce the side effects associated with antipsychotics.

Since the 5-HT2A receptor has been implicated in the mechanism of action of atypical antipsychotics, it has been hypothesized that the 5-HT2A receptor may play a role in the improvement of negative and cognitive symptoms seen in some patients taking atypical antipsychotics. There have been several studies focusing on the binding and effects of atypical antipsychotics such as olanzapine and clozapine of the 5-HT2A receptor (Kasper, Hale et al. 1999, Willins, Berry et al. 1999). Atypical antipsychotics act as antagonists of 5-HT2A/$G\alpha_{q/11}$ pathway and it was considered for some time that this could be contributing to the therapeutic effect (Kasper, Hale et al. 1999).

However, our laboratory found that olanzapine and clozapine not only blocked 5-HT2A/$G\alpha_{q/11}$ signaling, but also activated the JAK2/STAT3 pathway in neuronal cells (Muma, Singh et al. 2007, Singh, Shi et al. 2007, Singh, Dai et al. 2009). Previously, the 5-HT2A/JAK/STAT signaling pathway had only been studied in skeletal muscle and VSM using 5-HT to stimulate the JAK/STAT pathway. This novel signaling pathway in neuronal cells illustrates that atypical antipsychotics are functionally selective at the 5-HT2A receptor by antagonizing 5-HT2A/$G\alpha_{q/11}$ signaling and activating 5-HT2A/JAK2/STAT3. The long term goal of our research is to investigate the two different 5-HT2A receptor signaling pathways to

understand how atypical antipsychotics produce a therapeutic effect and improve the treatment for schizophrenia. Therefore the purpose of this study is to identify functionally selective ligands of the 5-HT2A receptor.

In order to study the 5-HT2A/$G\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 pathways separately, we need to identify ligands that are functionally selective for the 5-HT2A receptor. Olanzapine activates the 5-HT2A/JAK2/STAT3 pathway but antagonizes 5-HT2A/$G\alpha_{q/11.}$ Ketanserin, a 5-HT2A receptor antagonist, blocks both 5-HT2A/$G\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 pathways (Banes, Shaw et al. 2005). Therefore, we are using analogs of ketanserin and olanzapine to identify ligands that can be used to study the two pathways independently in the future. To study the 5-HT2A/$G\alpha_{q/11}$ pathway, we used an intracellular calcium assay. After treatment with the ligand, increases in intracellular calcium can be used as an index of activation of the 5-HT2A/$G\alpha_{q/11}$ pathway, while in decrease in DOI-induced intracellular calcium is indicative of antagonism of the 5-HT2A/$G\alpha_{q/11}$ pathway. To study the 5-HT2A/JAK2/STAT3 pathway, we used immunoblotting for phospho-JAK2. Activation of 5-HT2A/JAK2/STAT3 pathway by a ligand is indicated by an increase in phosphorylated JAK2, while antagonism of the 5-HT2A/JAK2/STAT3 pathway is indicated by decreased olanzapine-stimulated phosphorylation of JAK2.

Chapter 2: Materials and Methods

Drugs

Olanzapine and ketanserin-tartrate were purchased from Tocris Bioscience. Olanzapine was dissolved in 20% glacial acetic acid to make a 6mM stock solution and the pH was adjusted to 6.5 by using 10M NaOH. 6mM olanzapine was then diluted in media to a final concentration of 600nM. A stock solution of 2mM ketanserin was made in water. 2mM ketanserin was diluted in media or Kreb's medium to a final concentration of 10nM. DOI (2,5-dimethoxy-4-iodoamphetamine HCl) was purchased from Sigma. DOI was made up in water for a 6mM stock solution and diluted Kreb's medium to a final concentration of 12uM. Analogs of olanzapine and ketanserin were selected by Dr. Prisinzano's laboratory. 10mM stock solutions of the analogs were prepared in 100% DMSO and stored at 4C and then diluted in water to 1mM, immediately prior to use.

Drug Treatments

In previous studies, 10nM ketanserin was used to antagonize 5-HT2A signaling through both 5-HT2A/G$\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 (Banes, Shaw et al. 2005). Since the analogs were based on the structure of ketanserin, the analogs were tested at 1nM, 10nM, and 100nM concentrations. The 1mM stock solutions of analogs were diluted in Kreb's medium or cell culture media for final treatments. For the calcium mobilization assay, the baseline fluorescence was recorded for 30 seconds at 10 second intervals. Then the vehicle, DOI, or analogs were injected into the plate and the fluorescence response was recorded for 2 minutes at 10 second intervals to evaluate an intracellular calcium response. For the analogs that increased calcium mobilization, I determined whether the analogs are causing the response via the 5-HT2A receptor, by pretreatment for 30 minutes with ketanserin to block the response. For the analogs

that did not increase calcium mobilization, I determined whether the analogs could be antagonists of the 5-HT2A receptor by pretreatment with the analogs for 30 minutes and then stimulated with 12 μM DOI to investigate if the analog could block the DOI-induced increase in intracellular calcium.

For JAK2 phosphorylation assay, cells were treated with vehicle, olanzapine, or analogs for 24 hours before the assay was performed to investigate agonism of the 5-HT2A/JAK2/STAT3 pathway. For analogs that increased phosphorylation of JAK2, 30 minute pretreatment with ketanserin was used to block the analog induced phosphorylation of JAK2 in order to determine if the increase of phosphorylated JAK2 was due to activation of the 5-HT2A receptor. For analogs that did not produce an increase in phosphorylation of JAK2, the cells were pretreated with the analog for 30 minutes and then treated with olanzapine for 24 hours to determine whether any of the analogs could antagonize the olanzapine induced activation of the 5-HT2A/JAK2/STAT3 pathway.

Cell Culture

Clu119 cells, purchased from Cellutions Biosystems (Burlington, NC), are an embryonic mouse hypothalamus cell line that expresses 5-HT2A receptors. Clu119 cells were grown in Dulbecco's modified Eagle's medium containing glucose, L-glutamine, and sodium bicarbonate, with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Cells were grown at 37°C with 5% CO_2. All cells were grown in 10% charcoal-treated FBS to reduce the serotonin in the medium for 48 hours prior to being treated with drugs, harvested, or used in calcium mobilization assay (Unsworth and Molinoff 1992, Dai, Dudek et al. 2011).

Calcium Mobilization Assay

The calcium mobilization assay is a fluorescence based assay that utilizes Fura-2 AM as an index of intracellular calcium concentration. Activation of the 5-HT2A/$G\alpha_{q/11}$ pathway leads to activation of PLC which catalyzes the formation of IP3. IP3 binds to the IP3 receptor on the endoplasmic reticulum and leads to the release of calcium into the cytosol. When the calcium is released from the endoplasmic reticulum, it will bind to the Fura-2 AM and create a fluorescent response.

Cells were split into black-sided, clear bottom, 96-well plates. Cell were washed twice with 200µl Kreb's medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM D-glucose, 11.6 mM Hepes, pH 7.3) and then incubated with 200µl loading buffer (5 µM Fura-2 AM, 0.1% bovine serum albumin, and 0.02% Pluronic F127 in Kreb's medium) for 1hr at 37°C in the dark. Loading buffer was removed and cells were washed 2 times with 200µl Kreb's medium. The cells were then pretreated with drugs or Kreb's medium for 30 minutes at 37°C in the dark. The medium was replaced with 100µl fresh Kreb's medium. The plate was then placed in the BioTek Synergy 2 plate reader for injections of test compounds. The ratio of Fura-2 fluorescence at 340nm to 380nm excitation was measured using the BioTek Synergy 2 fluorescence plate reader. The background fluorescence was subtracted and this calculation was used as an index of intracellular calcium concentration. The area under the curve was calculated for all of the data points for 2 minutes and used to compare calcium mobilization.

JAK2 Phosphorylation Assay

To investigate the 5-HT2A/JAK2/STAT3 pathway, western blotting was performed using a phospho-JAK2 and total JAK2 antibodies as an index of activation of the 5-HT2A/JAK2/STAT3 pathway.

Harvesting

Cells were grown in 10cm plates and then harvested and separated into membrane and cytosolic fractions. Cells were collected in hypotonic buffer (0.25M sucrose, 50mM Tris-HCl, 5mM EDTA, 100mM NaCl, protease inhibitor cocktail, and phosphatase inhibitors). The cell lysate was sonicated to homogenize the samples and then spun down at 25000xg for 1hr at 4°C to collect the supernatant containing the cytosolic fraction and pellet the membrane fraction. The membrane fraction pellet was reconstituted using solubilization buffer (20mM Tris, 1mM EDTA, 100mM NaCl, 1% sodium cholate, protease inhibitor cocktail, and phosphatase inhibitors) and sonicated to break up the pellet. The centrifuge tubes were shaken horizontally for 1hr at 4°C. The homogenate was then centrifuged at 25000xg for 1hr at 4°C. The supernatant containing the membrane fraction was then collected, aliquoted, and stored at -80°C.

Western Blotting

A bicinchoninic acid assay was used to determine the total concentration of protein in each sample. Equal amounts of proteins of the membrane fractions were separated on 10% SDS-PAGE gels at 150V and transferred to polyvinylidene fluoride membranes at 100V for 2hrs. The membranes were incubated in 5% non-fat milk made in Tris-buffered saline, 0.1% Tween-20 (TBST) at room temperature for one hour to block non-specific binding. The membranes were then incubated in anti-JAK2 (1:1000, monoclonal antibody, #04-001, EMD Millipore, USA) overnight at 4°C. After washing 4 times for 10 minutes with TBST, the membranes were incubated in goat-anti-mouse secondary antibody (1:10000, #119380, Jackson ImmunoResearch, USA) at room temperature for 1hr. The membranes were washed 4 times for 10 minutes with TBST. The membranes were incubated with ECL, a chemiluminescence solution, (Cat# WBLUR0500, Millipore, USA, Cat# 10026384, Cat# 10026385, BioRad, USA) and the

chemiluminescent signal was captured using BioRad ChemiDoc XRS+ molecular imager (BioRad, USA).

The membranes were then incubated in 5% bovine serum albumin made in TBST and incubated in anti-phospho-JAK2 (1:1500, monoclonal antibody, #3779, Cell Signaling, USA) overnight at 4°C. Membranes were washed 3 times for 5 minutes with TBST and incubated in goat-anti-rabbit secondary antibody (1:10000, #120745, Jackson Immuno Research, USA) at room temperature for 1hr. The membrane was washed 3 times for 5 minutes and membranes were incubated with ECL and the data collected as described above.

The membranes were then incubated in anti-β-actin antibody (1:20000, #ICN691001, MP Biomedicals) for 30 minutes and washed 3 times for 10 minutes with TBST. The membranes were incubated in goat-anti-mouse antibody for an hour at room temperature and washed 3 times for 10 minute. The membranes were incubated with ECL reagent and the luminescence was detected.

The integrated optical density (IOD) of the bands was quantified and analyzed using ImageLab 3.0 software (BioRad, USA) and the pJAK2 was normalized to JAK2.

Data Analysis and Statistics

GraphPad Prism 5.0 (GraphPad Software Inc., USA) was used for all statistical analyses. A probability level of $p<0.05$ was considered to be statistically significant. For the calcium assay, AUC data was analyzed by a one-way ANOVA and presented as the mean ± the standard error of the mean. The data was then analyzed using the Tukey's post hoc test. For the JAK2 phosphorylation assay, data was presented as the mean ± the standard error of the mean and

analyzed by a one-way ANOVA. The data was then analyzed using the Tukey's or Dunnett's post hoc test.

Chapter 3: Results

Previously, our laboratory reported that olanzapine stimulated the 5-HT2A/JAK2/STAT3 pathway in rat frontal cortex, rat hypothalamus, and an embryonic rat cortical neuronal cell line, A1A1v cells (Muma, Singh et al. 2007, Singh, Dai et al. 2009). My initial studies focused on determining if Clu119 cells, an embryonic mouse hypothalamus cell line, could be used for studying the 5-HT2A/JAK2/STAT3 pathway. Clu119 cells express 5-HT2A receptors, STAT3, and JAK2, as well as $G\alpha_{q/11}$ proteins and other downstream effectors of the 5-HT2A/$G\alpha_{q/11}$ pathway (CedarLane, Burlington, NC). The calcium mobilization assay was used as an indicator of 5-HT2A/$G\alpha_{q/11}$ signaling because the release of intracellular calcium from the endoplasmic reticulum is a result of downstream effectors from $G\alpha_{q/11}$ proteins coupling to 5-HT2A receptors (Raote, Bhattacharya et al. 2007, Paredes, Etzler et al. 2008). Previous studies have reported that DOI increases calcium mobilization via the 5-HT2A receptor and that this increase could be blocked by ketanserin (Hoyer, Clarke et al. 1994, Dai, Dudek et al. 2011). Clu119 cells were treated with vehicle or 12uM DOI, with or without a 30 minute pretreatment with 10nM ketanserin (Fig. 1A). An area under the curve (AUC) analysis of the fluorescence ratio over time indicates that 12uM DOI significantly increased calcium mobilization and pretreatment with ketanserin blocked the DOI-induced calcium mobilization (Fig. 1B). Immunoblotting was used to investigate the phosphorylation of JAK2 as an indicator of 5-HT2A/JAK2/STAT3 signaling. Cells were treated with 600nM olanzapine for 24 hours. Treatment with olanzapine induced phosphorylation of JAK2 which was blocked with 30 minute pretreatment with 10nM ketanserin (Fig 1C). Based on these results, Clu119 cells were used to investigate the 5-HT2A/$G\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 pathway.

Analogs that are structurally similar to olanzapine and ketanserin were selected from a library to investigate functional selectivity of the 5-HT2A receptor. SCAP109608 (608), SCAP109609 (609), SCAP109610 (610), SCAP109615 (615), and SCAP109618 (618) were the five analogs selected to determine their effects on both the 5-HT2A/$G\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 pathways. The analogs were tested at three different concentrations: 1nM, 10nM, and 100nM. These concentrations were selected based on previous experiments that demonstrated that ketanserin blocked 5-HT2A receptor signaling at the 10nM concentration (Banes, Shaw et al. 2005).

Identifying Agonists of the 5-HT2A/$G\alpha_{q/11}$ Pathway

Clu119 cells were plated in 96 well plates and treated with Fura-2-AM, which binds to intracellular calcium and produces a fluorescent signal. The cells were then treated with 1nM, 10nM, and 100nM concentrations of 608, 609, 610, 615, and 618. AUC analysis indicates there was no significant difference between treatments of 608 and the vehicle (Fig 2A & 2B). Treatment of the cells with 10nM 610 caused significantly increased intracellular calcium compared to the vehicle. There was no significant difference between the vehicle and treatment with 1nM and100nM 610. Pretreatment with ketanserin significantly reduced the calcium response to 10nM 610 (Fig. 2C & 2D). There was a significant increase in intracellular calcium when cells were treated with 1nM and 100nM 618. However, there was no significant decrease when cells were pretreated with ketanserin and then treated with 100nM 618 (Fig. 2E & 2F). Treatment with 609 and 615 did not induce calcium mobilization (Fig. 3A, 3C, 3E, & 3G).

Identifying Antagonists of the 5-HT2A/$G\alpha_{q/11}$ Pathway

Neither 609 nor 615 increased the fluorescence ratio which indicates that neither activate the 5-HT2A/$G\alpha_{q/11}$ pathway. Therefore, we investigated the possibility that 609 and 615 could

antagonize the 5-HT2A/$G\alpha_{q/11}$ pathway. Clu119 cells were pretreated with 100nM of 609 or 615 and then stimulated with DOI to determine if either analog could block DOI-induced calcium mobilization (Fig. 3B & 3F). AUC analysis indicates that both 609 and 615 significantly reduced the DOI-induced calcium mobilization (Fig. 3D & 3H).

Effect of Analogs on the 5-HT2A/JAK2/STAT3 Pathway

Clu119 cells were treated with 1nM, 10nM, 100nM conentrations of 608, 609, 610, 615, 618, vehicle, or 600nM olanzapine for 24 hours before harvesting. Antibodies for both pJAK2 and JAK2 were used to analyze the treatment effect. The bar graphs represent the quantification of pJAK2 divided by the JAK2. After 24 hours treatment with 100nM 608, pJAK2 was significantly increased compared to the vehicle, but there was no significant increase with 1nM or 10nM 608. Pretreatment with ketanserin did not significantly decrease the stimulation of pJAK2 by 100nM 608 (Fig 4A). Treatment with 1nM 610 significantly increased pJAK2 compared to the vehicle, but there was no significant increase with 10nM or 100nM 610. Pretreatment with ketanserin did not significantly decrease the 1nM 610 stimulation of pJAK2 (Fig 4B). 1nM 618 significantly increased pJAK2 compared to vehicle and when experiments were repeated with pretreatment with vehicle or ketanserin, 10nM 618 also significantly increaed pJAK2. There was no signficant antagonization of 10nM 618 when pretreated with ketanserin (Fig 4C). When the cells were pretreated with vehicle or ketanserin and then treated with 10nM 609, treatment with 10nM 609 significantly increased pJAK2. Pretreatment with ketanserin significantly reduced the 10nM 609 stimulated increase of pJAK2 (Fig 5A). Treatment with 615 did not increase pJAK2. Therefore, we investigated the posibility that 615 could be an antagonist of the 5-HT2A/JAK2/STAT3 pathway. Cells were pretreated with 100nM 615 for 30 minutes

and then treated with 600nM olanzapine for 24 hours. Pretreatment with 100nM 615 significantly reduced the olanzapine stimulated pJAK2 (Fig 5B).

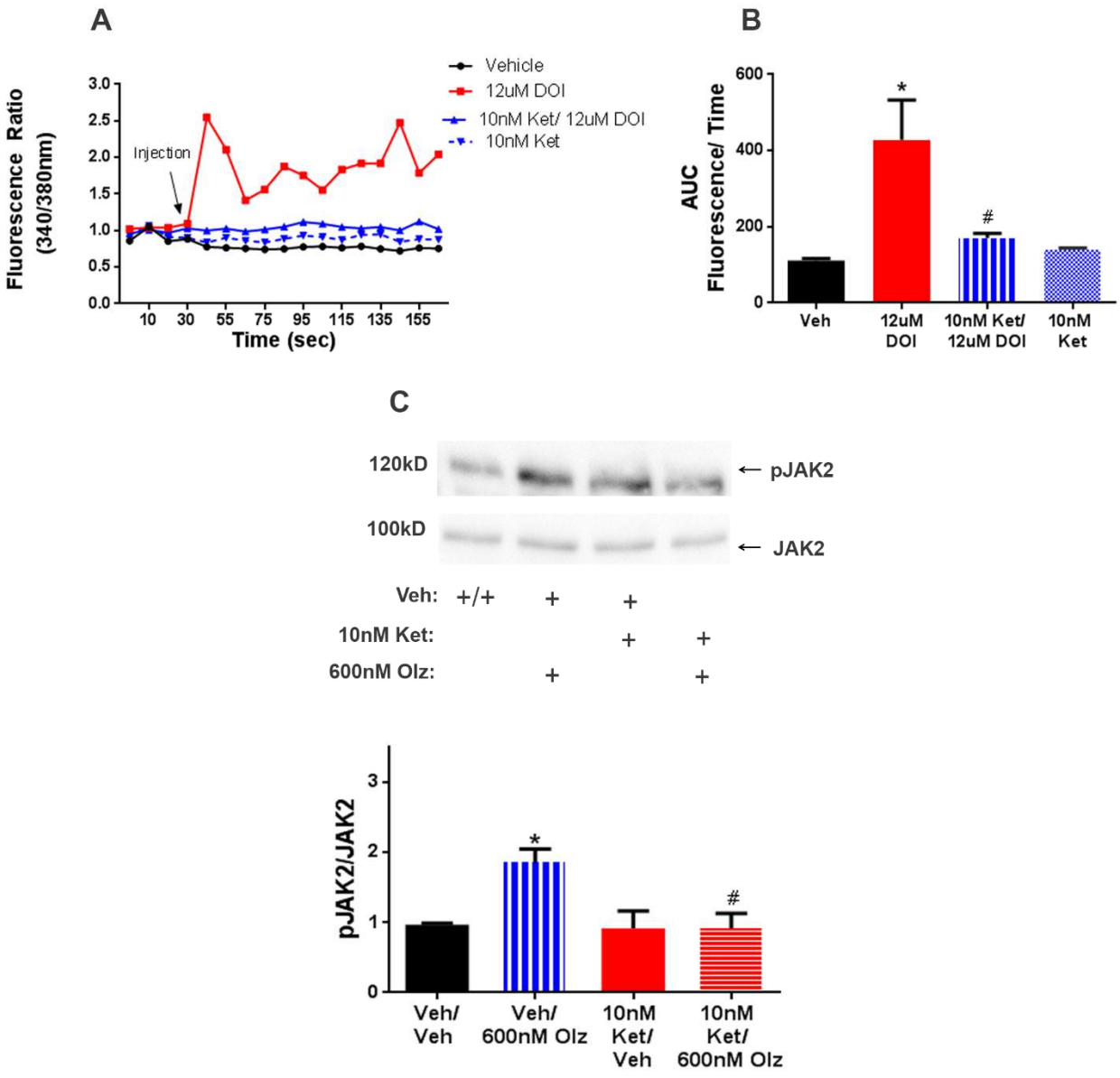

Figure 1: DOI stimulates the 5-HT2A/G$\alpha_{q/11}$ and olanzapine stimulates the 5-HT2A/JAK2/STAT3 pathway in Clu119 cells. **A** Clu119 cells were treated with vehicle or 12uM DOI, with or without 10 nM ketanserin pretreatment. **B** For AUC, one-way ANOVA indicates significance between treatments [$F(3,8)= 7.793$; $p<0.01$]. Tukey's post hoc analysis reports *$p<0.05$ between vehicle and 12uM DOI and #$p<0.05$ between 12uM DOI and 10nM ketanserin/

12uM DOI. **C** Cells were pretreated with vehicle or 10nM ketanserin for 30 minutes and then treated with vehicle or 600nM olanzapine for 24 hours. One-way ANOVA indicates significance between treatment [$F(3,14)=8.980$; $p<0.005$]. Tukey's post hoc analysis reports *$p<0.005$ between vehicle/vehicle and vehicle/600nM olanzapine and #$p<0.01$ between vehicle/600nM olanzapine and 10nM ketanserin/600nM olanzapine.

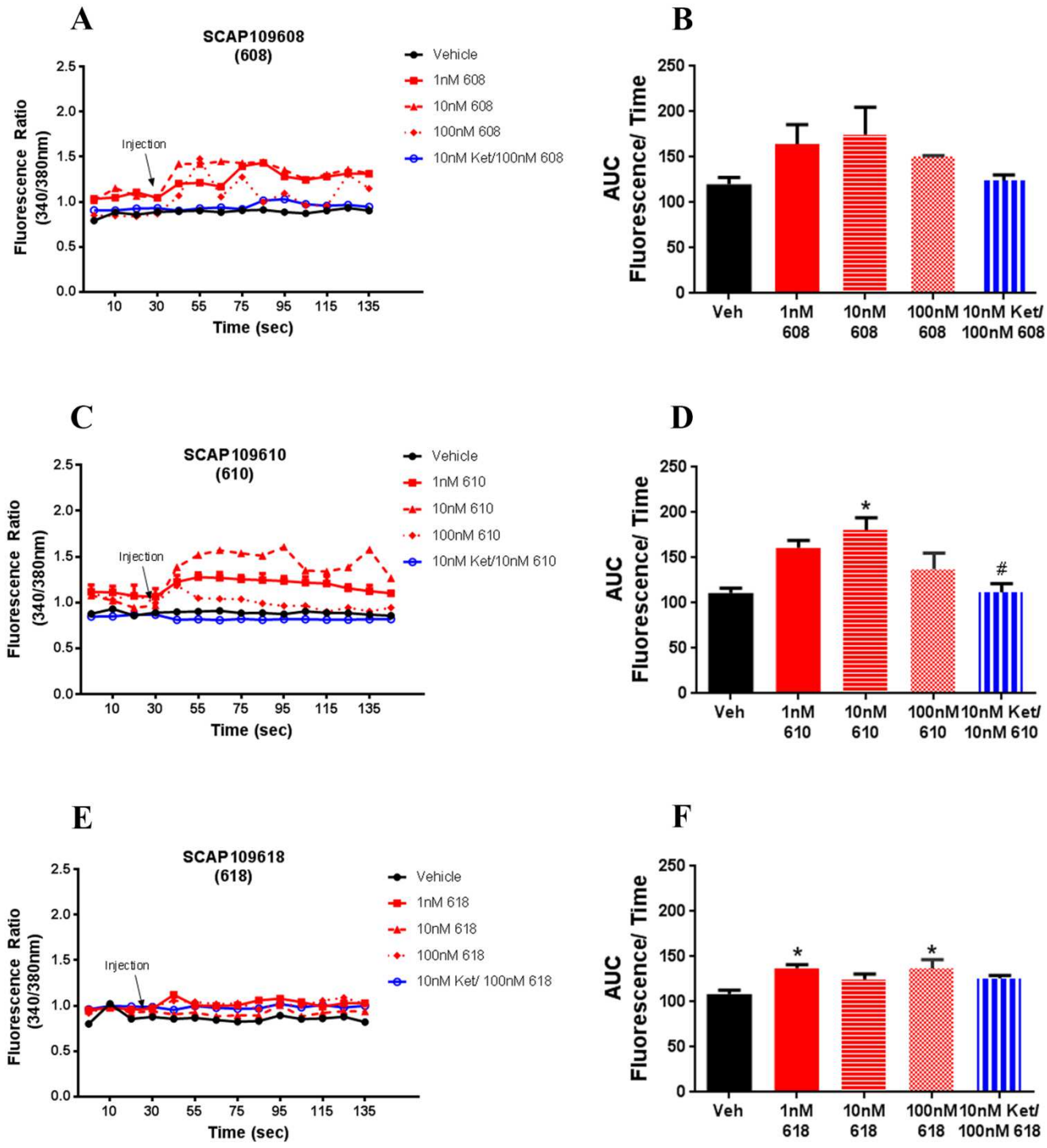
A
SCAP109608
(608)
Vehicle
1nM 608
10nM 608
100nM 608
10nM Ket/100nM 608
Injection
Fluorescence Ratio
(340/380nm)
Time (sec)
B
AUC
Fluorescence/ Time
Veh
1nM 608
10nM 608
100nM 608
10nM Ket/ 100nM 608
C
SCAP109610
(610)
Vehicle
1nM 610
10nM 610
100nM 610
10nM Ket/10nM 610
Injection
Fluorescence Ratio
(340/380nm)
Time (sec)
D
AUC
Fluorescence/ Time
*
#
Veh
1nM 610
10nM 610
100nM 610
10nM Ket/ 10nM 610
E
SCAP109618
(618)
Vehicle
1nM 618
10nM 618
100nM 618
10nM Ket/ 100nM 618
Injection
Fluorescence Ratio
(340/380nm)
Time (sec)
F
AUC
Fluorescence/ Time
*
*
Veh
1nM 618
10nM 618
100nM 618
10nM Ket/ 100nM 618

Figure 2: Effect of analogs through the 5-HT2A/$G\alpha_{q/11}$ pathway. Clu119 cells were treated with vehicle or analogs and changes in 340/380nm fluorescence ratio were recorded for 2 minutes. Graphs represent mean of each data set and the mean ± the standard error of the mean of the area under the curve (n=3). The arrow represents the time of injection (30 seconds). **A** Cells were treated with 1nM, 10nM, and 100nM concentrations of 608, vehicle, and pretreatment with 10nM ketanserin, followed by treatment with 100nM 608. **B** For the AUC, there was no significant difference between treatments [$F(4,10)=1.992$; $p=0.171$]. **C** Cells were treated with 1nM, 10nM, and 100nM concentrations of 610, vehicle and pretreatment with ketanserin, followed by treatment with 10nM 610. **D** For the AUC, one-way ANOVA indicates that there is a significant difference between treatments [$F(4, 10)=6.684$; $p<0.01$] and Tukey's post hoc test reported *$p<0.05$, n=3 between vehicle and 10nM 610 and #$p<0.05$, n=3 between 10nM 610 and 10nM Ket/10nM 610. **E** Cells were treated with 1nM, 10nM, and 100nM concentrations of 618, vehicle and pretreatment with ketanserin, followed by treatment with 100nM 618. **F** For the AUC, one-way ANOVA indicates that there is a significant difference between treatments [$F(4, 10)=3.779$; $p<0.05$] and Tukey's post hoc test reported *$p<0.05$, n=3 between vehicle and 1nM 618 and vehicle and 100nM 618.

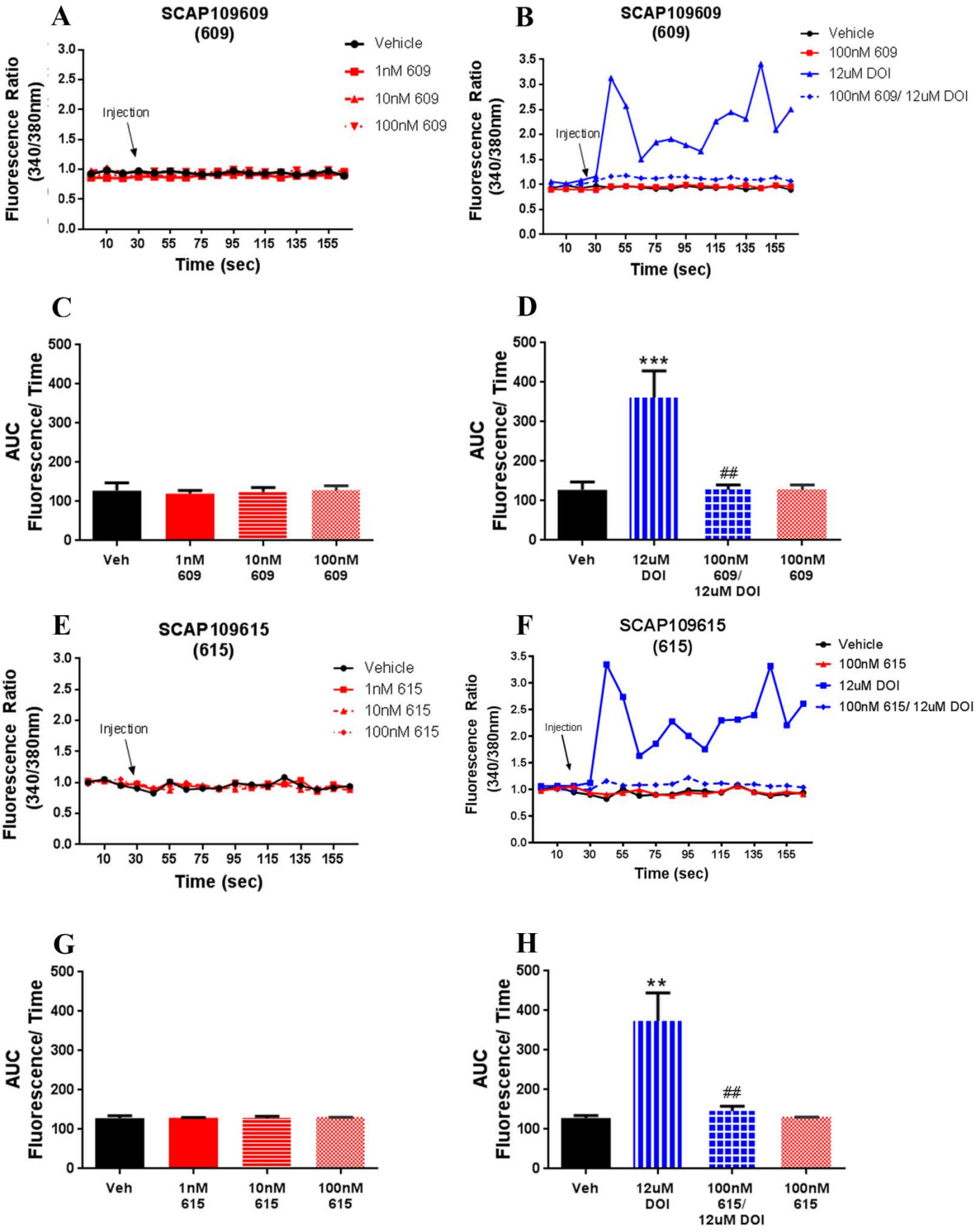
A
SCAP109609
(609)
Vehicle
1nM 609
10nM 609
100nM 609
Injection
Fluorescence Ratio
(340/380nm)
Time (sec)
B
SCAP109609
(609)
Vehicle
100nM 609
12uM DOI
100nM 609/ 12uM DOI
Injection
Fluorescence Ratio
(340/380nm)
Time (sec)
C
AUC
Fluorescence/ Time
Veh
1nM 609
10nM 609
100nM 609
D
AUC
Fluorescence/ Time
***
##
Veh
12uM DOI
100nM 609/ 12uM DOI
100nM 609
E
SCAP109615
(615)
Vehicle
1nM 615
10nM 615
100nM 615
Injection
Fluorescence Ratio
(340/380nm)
Time (sec)
F
SCAP109615
(615)
Vehicle
100nM 615
12uM DOI
100nM 615/ 12uM DOI
Injection
Fluorescence Ratio
(340/380nm)
Time (sec)
G
AUC
Fluorescence/ Time
Veh
1nM 615
10nM 615
100nM 615
H
AUC
Fluorescence/ Time
**
##
Veh
12uM DOI
100nM 615/ 12uM DOI
100nM 615

Figure 3: Effect of analogs through the 5-HT2A/$G\alpha_{q/11}$ pathway and DOI-induced calcium mobilization. Clu119 cells were treated with vehicle or analogs and changes in 340/380nm fluorescence ratio were recorded for 2 minutes. The arrow represents the time of injection (30 seconds). Graphs represent the mean ± the standard error of the mean of the area under the curve (n=3). **A** Cells were treated with 1nM, 10nM, and 100nM 609, or vehicle. **B** Cells were treated with vehicle, 100nM 609, and 12μM DOI; with and without pretreatment with 100nM 609. **C** AUC analysis indicates no significant differences between 1nM, 10nM, 100nM 609 and vehicle. **D** For AUC analysis, one-way ANOVA indicates that there is a significant difference between treatment groups [$F(3, 8)=10.47$; $p<0.005$] and Tukey's post hoc test reported **$p<0.01$, n=3 for 12uM DOI treatment compared to vehicle and ##$p<0.01$, n=3 for 100nM 609/12uM DOI compared to 12uM DOI. **E** Cells were treated with 1nM, 10nM, and 100nM 615. **F** Cells were injected with vehicle, 100nM 615, and 12μM DOI; with and without pretreatment with 100nM 615. **G** AUC analysis indicates no significant differences between 1nM, 10nM, 100nM 615 and vehicle. **H** For AUC analysis, one-way ANOVA indicates significance between treatment groups [$F(3, 8)=11.07$; $p<0.005$] and Tukey's post hoc test reported **$p<0.01$, n=3 for 12uM DOI treatment compared to vehicle and ##$p<0.01$, n=3 for 100nM 615/12uM DOI compared to 12uM DOI.

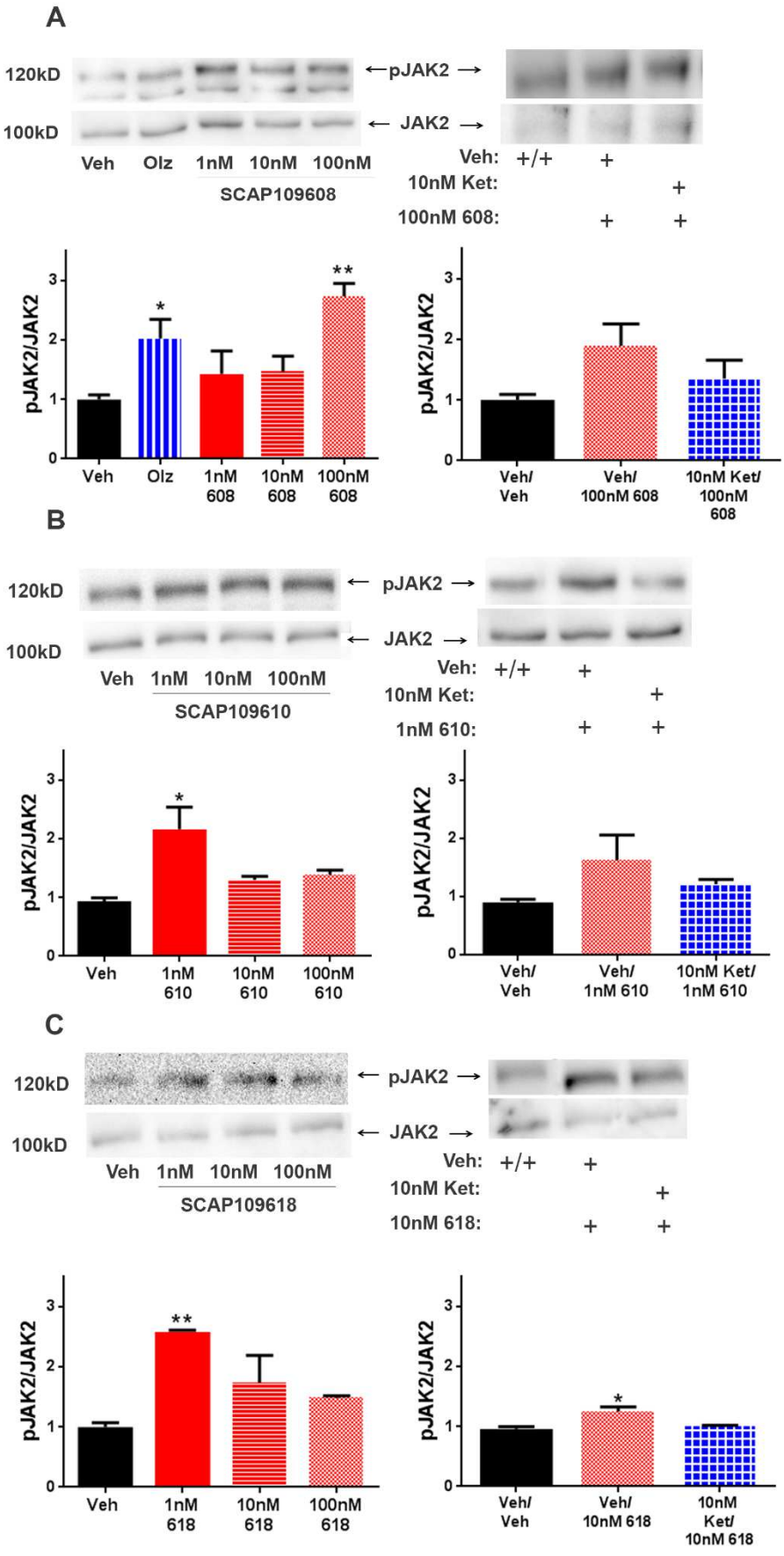
A
120kD
100kD
pJAK2
JAK2
Veh Olz 1nM 10nM 100nM
SCAP109608
Veh: +/+ + +
10nM Ket: +
100nM 608: + +
pJAK2/JAK2
Veh Olz 1nM 608 10nM 608 100nM 608
Veh/ Veh
Veh/ 100nM 608
10nM Ket/ 100nM 608
B
120kD
100kD
pJAK2
JAK2
Veh 1nM 10nM 100nM
SCAP109610
Veh: +/+ +
10nM Ket: +
1nM 610: + +
pJAK2/JAK2
Veh 1nM 610 10nM 610 100nM 610
Veh/ Veh
Veh/ 1nM 610
10nM Ket/ 1nM 610
C
120kD
100kD
pJAK2
JAK2
Veh 1nM 10nM 100nM
SCAP109618
Veh: +/+ +
10nM Ket: +
10nM 618: + +
pJAK2/JAK2
Veh 1nM 618 10nM 618 100nM 618
Veh/ Veh
Veh/ 10nM 618
10nM Ket/ 10nM 618

Figure 4: Effect of analogs through the 5-HT2A/JAK2/STAT3 pathway. Clu119 cells were treated with vehicle or analogs for 24 hours or pretreated with vehicle or ketanserin for 30 minutes and then treated with vehicle or analogs for 24 hours. Antibodies for both pJAK2 and JAK2 were used to analyze the results. The bar graphs represent the mean quantification of pJAK2 divided by the JAK2 (n=3). **A** Cells were treated with 1nM, 10nM, and 100nM concentrations of 608, olanzapine, or vehicle for 24 hours (left) or cells were pretreated with vehicle or ketanserin for 30 minutes and then treated with vehicle or 100nM 608 (right). For the left graph, one-way ANOVA indicates that there is a significant difference between treatment groups [$F(3, 14)=4.717$; $p<0.05$] and Dunnett's post hoc test reported $^{**}p<0.01$ for 100nM 608 compared to vehicle. **B** Cells were treated with 1nM, 10nM, and 100nM concentrations of 610, or vehicle for 24 hours (left) or cells were pretreated with vehicle or ketanserin for 30 minutes and then treated with vehicle or 1nM 610 (right). For the left graph, one-way ANOVA indicates that there is a significant difference between treatment groups [$F(4, 13)=3.387$; $p<0.05$] and Dunnett's post hoc test reported $^{*}p<0.05$, n=3 for 1nM 610 treatment compared to vehicle. **C** Cells were treated with 1nM, 10nM, and 100nM concentrations of 618, or vehicle for 24 hours (left) or cells were pretreated with vehicle or ketanserin for 30 minutes and then treated with vehicle or 10nM 618 (right). For the left graph, one-way ANOVA indicates that there is a significant difference between treatment groups [$F(4, 8)=7.756$; $p<0.01$] and Dunnett's post hoc test reported $^{**}p<0.005$, n=2 for 10nM 610 treatment compared to vehicle. For the right graph, one-way ANOVA analysis indicates a significant difference between treatment groups [$F(2,5)=6.910$; $p<0.05$] and Tukey's post hoc test reported $^{*}p<0.05$, n=3 for vehicle/10nM 618 compared to vehicle/vehicle.

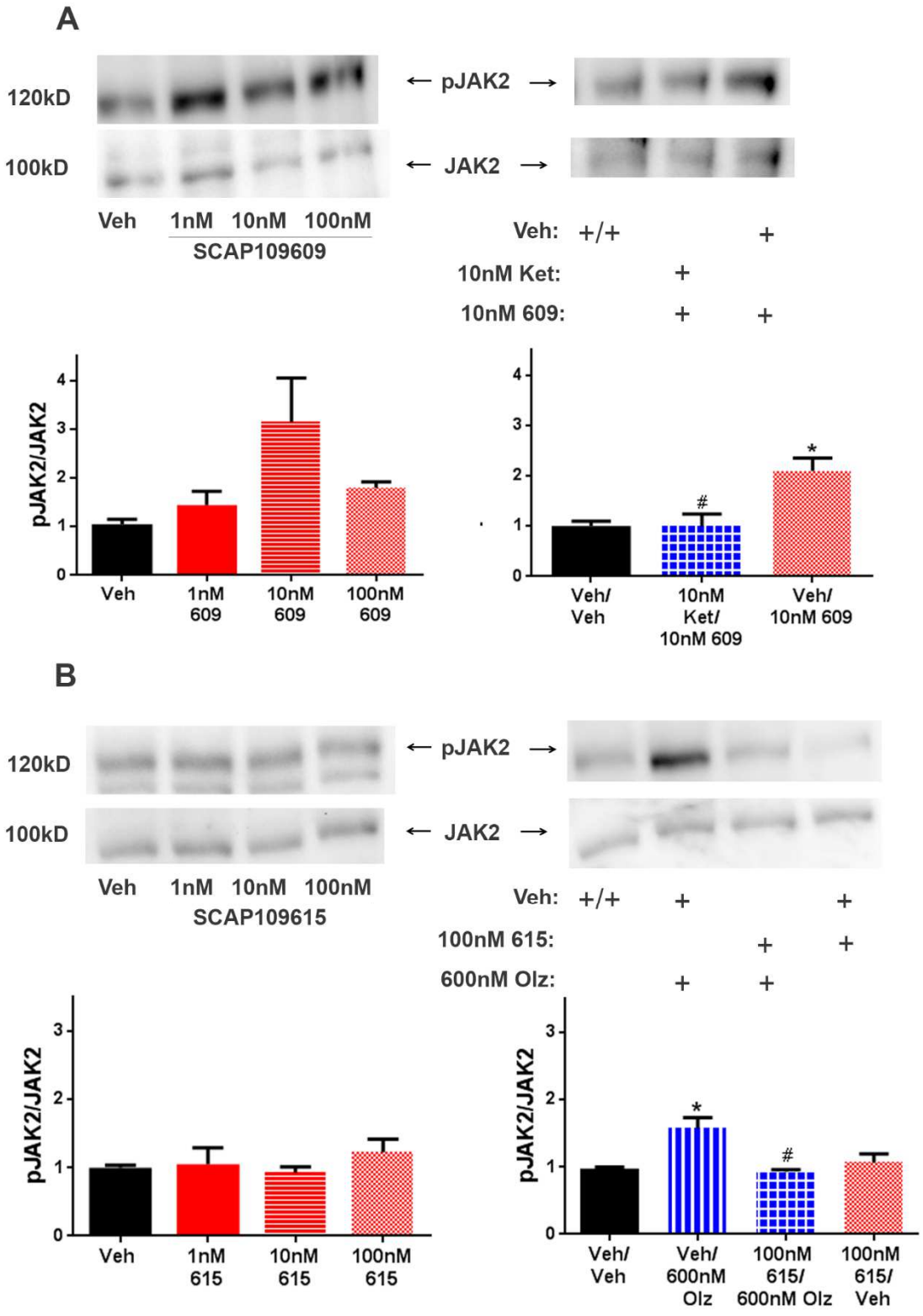
A
120kD
100kD
← pJAK2 →
← JAK2 →
Veh 1nM 10nM 100nM
SCAP109609
Veh: +/+ +
10nM Ket: +
10nM 609: + +
pJAK2/JAK2
Veh
1nM 609
10nM 609
100nM 609
Veh/ Veh
10nM Ket/ 10nM 609
Veh/ 10nM 609
B
120kD
100kD
← pJAK2 →
← JAK2 →
Veh 1nM 10nM 100nM
SCAP109615
Veh: +/+ + +
100nM 615: + +
600nM Olz: + +
pJAK2/JAK2
Veh
1nM 615
10nM 615
100nM 615
Veh/ Veh
Veh/ 600nM Olz
100nM 615/ 600nM Olz
100nM 615/ Veh

Figure 5: Effect of analogs through the 5-HT2A/JAK2/STAT3 pathway. Clu119 cells were treated with vehicle or analogs for 24 hours or pretreated with vehicle or ketanserin for 30 minutes and then treated with vehicle or analogs for 24 hours. Antibodies for both pJAK2 and JAK2 were used to analyze the results. The bar graphs represent the mean quantification of pJAK2 divided by the JAK2 (n=3). **A** Cells were treated with 1nM, 10nM, and 100nM concentrations of 609, or vehicle for 24 hours or cells were pretreated with vehicle or ketanserin for 30 minutes and then treated with vehicle or 10nM 609. For the right graph, one-way ANOVA analysis indicates a significant difference between treatment groups [$F(2,5)=9.957$; $p<0.05$] and Tukey's post hoc test reported *$p<0.05$, n=3 for vehicle/10nM 609 compared to vehicle/vehicle and #$p<0.05$, n=3 for vehicle/10nM 609 compared to 10nM ketanserin/10nM 609. **B** Cells were treated with 1nM, 10nM, and 100nM concentrations of 615, or vehicle for 24 hours or cells were pretreated with vehicle or 100nM 615 for 30 minutes and then treated with vehicle or 600nM olanzapine. One-way ANOVA analysis of the pretreatment with 100nM 615 indicates a significant difference between treatment groups [$F(3,7)=6.852$; $p<0.05$]. Tukey's post hoc test reported *$p<0.05$, n=3 for vehicle/600nM olanzapine compared to vehicle/vehicle and #$p<0.05$, n=3 for 100nM 615/600nM olanzapine compared to vehicle/600nM olanzapine.

Chapter 4: Discussion

GPCRs such as serotonin, μ-opioid, β2-adrenergic, angiotensin receptors, V2 vasopressin, and dopamine receptors, have become targets for drug discovery, because ligands are often functionally selective at these receptors (Mottola, Laiter et al. 1996, Yu, Zhang et al. 1997, He, Fong et al. 2002). Targeting specific signaling pathways through a receptor can lead to more effective treatment options and possibly lead to a better understanding of different disorders. FDA approved drugs often target members of a GPCR gene family, because GPCRs can couple to multiple G-proteins, arrestins, or other downstream effectors (Rankovic, Brust et al. 2016). These pathways can often be pharmacologically separated and therefore are being explored as new possibilities for GPCR-targeted drug discovery (Marti-Solano, Iglesias et al. 2015, Rankovic, Brust et al. 2016).

For example, 'beta-blockers', commonly used to treat myocardial infarction , are typically antagonists for β-adrenergic receptors, which couple to $G_{\alpha s}$-proteins, but also activate ERK signaling through β-arrestins (Azzi, Charest et al. 2003, Kenakin 2011, Whalen, Rajagopal et al. 2011). Angiotensin type 1 receptors are drug targets for treating hypertension and cardiovascular disorders. Certain drugs are unable to activate receptor coupling to G-proteins, but can also activate ERK phosphorylation through β-arrestins (Wei, Ahn et al. 2003). However, sometimes functionally selective ligands used to treat disorders can lead to unwanted side effects. Famotidine targets the histamine H2 receptor to treat gastrointestinal ulcers by acting as an inverse agonist for G_s-protein activation of adenylyl cyclase. Famotidine also activates the expression of histidine decarboxylase independently of G_s-proteins that lead to a rebound of acid hypersecretion after withdrawal (Alonso, Zappia et al. 2015). Therefore, there is still a need for more selective drugs.

This is also true for using atypical antipsychotics for the treatment of schizophrenia. Atypical antipsychotics have previously been reported to antagonize the 5-HT2A/$G\alpha_{q/11}$ signaling pathway and that this effect could contribute to improving management of the symptoms of schizophrenia (Meltzer, Li et al. 2003, Amato 2015). Our lab found that, in addition to antagonizing 5-HT2A/$G\alpha_{q/11}$, atypical antipsychotics olanzapine and clozapine also activate the 5-HT2A/JAK2/STAT3 pathway (Singh, Shi et al. 2007, Singh, Dai et al. 2009). Therefore, we are investigating the functional selectivity of ligands of the 5-HT2A receptor in order to better understand the effect of atypical antipsychotics at the 5-HT2A receptor and provide avenues of investigation for creating more effective treatments for schizophrenia. In the future, we hope to identify compounds that either antagonize 5-HT2A/$G\alpha_{q/11}$ signaling or activate 5-HT2A/JAK2/STAT3 signaling independently. These compounds can be used to study how atypical antipsychotics are producing a therapeutic effect for positive, negative, and cognitive symptoms.

Our lab used analogs of olanzapine and the 5-HT2A receptor antagonist ketanserin based on the previous data that olanzapine exhibited functional selectivity at the 5-HT2A receptor and ketanserin acts as an antagonist to both 5-HT2A/$G\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 pathways (Banes, Shaw et al. 2005, Singh, Shi et al. 2007). Analogs SCAP109608, SCAP109609, SCAP109610, SCAP109615, and SCAP109618 were tested to determine how they effected the 5-HT2A/$G\alpha_{q/11}$ and 5-HT2A/JAK2/STAT3 pathways.

Using the calcium mobilization assay, we identified three analogs that induced calcium mobilization and two analogs that prevented 5-HT2A receptor agonist-induced calcium mobilization. 608 did not significantly increase calcium mobilization, but both 1nM and 10nM concentrations showed an increasing trend. 610 significantly induced calcium mobilization at

10nM, but not at 100nM. Ketanserin blocked the effect by 10nM 610, indicating that 610 is activating the 5-HT2A/$G\alpha_{q/11}$ pathway. 5-HT2A receptors internalize when over stimulated, so it is possible that 100nM 610 is desensitizing the 5-HT2A receptors or has a toxic effect at this dosage (Raote, Bhattacharyya et al. 2013). 618 induced calcium mobilization at 1nM and 100nM. A more extensive dose response experiment with more replications should be done to determine the most effective concentration that 618 induces calcium mobilization, as well as to see if ketanserin can effectively block 618. However, the decrease in calcium mobilization after pretreatment with ketanserin and then treatment with 100nM 618 is not significant. Although treatment with 618 induced a significant increase in calcium mobilization, the increase was small, which may indicate that 618 is a partial agonist. This could account for why pretreatment with ketanserin did not significantly block the effects of 618. A higher dose of ketanserin could be used or an increase of replicates could help to determine if 618 is acting through the 5-HT2A/ $G\alpha_{q/11}$ pathway. Analogs 609 and 615 did not induce calcium mobilization over the vehicle. Therefore, we used these analogs to pretreat the cells for 30 minutes before DOI treatment. Both 609 and 615 significantly reduced the DOI- induced calcium mobilization, indicating that these analogs block the 5-HT2A/$G\alpha_{q/11}$ pathways.

Using immunoblotting to study the phosphorylation of JAK2, we identified four analogs that stimulated phosphorylation of JAK2 and one analog that prevented 5-HT2A receptor agonist-induced phosphorylation of JAK2. 100nM 608, 1nM 610, and 1nM and 10nM 618 significantly increased phosphorylated JAK2 over that of the vehicle, but pretreatment with ketanserin did not significantly reduce the phosphorylation of JAK2. There is a trend that ketanserin may be reducing the analog induced phosphorylation of JAK2, so more replicates are needed determine if 608, 610, and 618 are increasing phosphorylation of JAK2 through the 5-

HT2A/JAK2/STAT3 pathway. There is very little response with 1nM and 10nM 608, so concentrations higher than 100nM should be investigated and then ketanserin should be used to determine if phosphorylation of JAK2 is occurring through the 5-HT2A receptor. On the other hand, both 610 and 618 were more effective at the lower concentration, so these analogs should be investigated at lower concentrations. 10nM 609 significantly increased phosphorylation of JAK2 and this increase was blocked by ketanserin, indicating that the response by 609 is due to the 5-HT2A/JAK2/STAT3 receptor. 615 did not increase the phosphorylation of JAK2, but when cells were pretreated with 100nM 615 and then treated with olanzapine, the increase in phosphorylated JAK2 was significantly reduced, indicating that 615 is an antagonist of the 5-HT2A/JAK2/STAT3 receptor.

Analog	**Calcium Mobilization**	Antagonism by Ketanserin?	Antagonism of DOI Stimulation?	**JAK2 Phosphorylation**	Antagonism by Ketanserin?	Antagonism of Olanzapine Stimulation?
SCAP109608 "608"	Increase #	Yes #	_____	Increase	Yes #	_____
SCAP109609 "609"	No Effect	_____	Yes	Increase	Yes	_____
SCAP109610 "610"	Increase	Yes	_____	Increase	Yes #	_____
SCAP109615 "615"	No Effect	_____	Yes	No Effect	_____	Yes
SCAP109618 "618"	Increase	Yes #	_____	Increase	Yes #	_____

Table 1: Summary of the effects of the analogs on both the calcium mobilization assay and immunoblotting for JAK2 phosphorylation. The ability of the effect of each analog to either be antagonized by ketanserin or antagonize the stimulation by a known 5-HT2A receptor agonist is also presented. # represents results that were not significantly different, but indicated a trend.

Like olanzapine and clozapine, 609 antagonizes the 5-HT2A/$G\alpha_{q/11}$ pathway, but activates the 5-HT2A/JAK2/STAT3 pathway. This study found that ketanserin and 615 antagonize olanzapine-induced phosphorylation of JAK2 in Clu119 cells. Before this study, ketanserin was a known antagonist of the 5-HT2A/$G\alpha_{q/11}$ pathway and 5-HT2A/JAK2/STAT3 in vascular smooth muscle (Banes, Shaw et al. 2005). 610 is an agonist of the 5-HT2A/$G\alpha_{q/11}$ pathway and is a potential agonist of the 5-HT2A/JAK2/STAT3 receptor. Based on the results, a concentration between 10nM and 100nM 610 may activate that 5-HT2A/$G\alpha_{q/11}$ pathway, but not affect the 5-HT2A/JAK2/STAT3 pathway. 608 and 618 increase calcium mobilization and phosphorylation of JAK2, but were not significantly blocked by ketanserin. If these analogs are not acting through the 5-HT2A receptor, they could be acting through other receptors that activate JAK/STAT signaling, including type II cytokine receptors, interleukin receptors, erythropoietin receptors, and thrombopoietin receptors (Rawlings, Rosler et al. 2004, Nicolas, Amici et al. 2013).

Previously, compounds with similar structures, such as the class of phenylisopropylamines, have shown functional selectivity of the 5-HT2A receptor (Kurrasch-Orbaugh, Watts et al. 2003, Moya, Berg et al. 2007). DOI, 2,5-dimethoxy-4-methyl (DOM), and 4-bromo-2,5-dimethoxyphenylisopropylamine (DOB) differentially activate the 5-HT2A/$G\alpha_{q/11}$ or 5-HT2A/PLA2/AA pathways (Moya, Berg et al. 2007). DOM preferentially stimulated the 5-HT2A/PLA2/AA pathway while DON activated the 5-HT2A/$G\alpha_{q/11.}$ Serotonin activates 5-HT2A/$G\alpha_{q/11,}$ 5-HT2A/PLA2/AA, and 5-HT2A/JAK/STAT, while tryptamine is a full agonist for 5-HT2A/$G\alpha_{q/11}$ signaling and a weak partial agonist for the 5-HT2A/PLA2/AA pathway

(Kurrasch-Orbaugh, Watts et al. 2003). Our data is consistent with these reports that compounds with similar structures are functionally selective at the 5-HT2A receptor.

The 5-HT2A/JAK/STAT pathway has been previously studied in myoblasts from rat muscle fibers, vascular muscle cells, cortical cell lines, and rat hypothalamus and frontal cortex (Banes, Shaw et al. 2005, Muma, Singh et al. 2007, Singh, Shi et al. 2007). The 5-HT2A/$G\alpha_{q/11}$ pathway has been studied in various regions of the brain and peripheral systems that express 5-HT2A receptors. We chose to examine the functional selectivity of 5-HT2A receptors in a mouse hypothalamic cell line, Clu119, which expresses 5-HT2A receptors, STAT3, and JAK2, but not 5-HT2C. Clu119 cells also express 5-HT1B, estrogen receptors α and β, insulin-like growth factor receptors, brain derived neurotrophic factor (Biosystems). The analogs tested in this dissertation should be investigated in primary neurons to corroborate the results presented here.

In this dissertation, the calcium assay is used to determine action of the analogs on the 5-HT2A receptors. Other ways of studying the 5-HT2A/$G\alpha_{q/11}$ pathway include PLC activity, GTPγS binding assay, and IP3 accumulation assay. To be confident that the calcium mobilization seen in our experiments was due to 5-HT2A/$G\alpha_{q/11}$ signaling, we used ketanserin to antagonize 5-HT2A/$G\alpha_{q/11}$ signaling. However, a second antagonist should be used to confirm selectivity through the 5-HT2A receptors. Also, a second test for 5-HT2A/$G\alpha_{q/11}$ signaling, such as an IP3 accumulation assay, can be used to confirm the calcium assay data. The GTPγS binding assay can be used to investigate the effect the analogs have on 5-HT2A receptors coupling with $G\alpha_{q/11}$ proteins.

We used pJAK2 and JAK2 antibodies for immunoblotting to investigate the 5-HT2A/JAK2/STAT3 pathway. Although in VSM and skeletal muscle the 5-HT2A receptor activated JAK1, JAK2, STAT1, and STAT3 receptors, our previous data in rat cortical cells

showed that olanzapine induced increased JAK2 and STAT3, but not STAT1 and STAT5 (Banes, Shaw et al. 2005, Singh, Shi et al. 2007). These differences between cell types is another form of functional selectivity, but the analogs should be tested with other JAK/STATs to be certain that the 5-HT2A receptor is acting through JAK2/STAT3. Other receptors that can lead to activation of JAK/STAT signaling include type II cytokine receptors, interleukin receptors, erythropoietin receptors, and thrombopoietin receptors (Rawlings, Rosler et al. 2004, Nicolas, Amici et al. 2013). It is not known if any of these receptors are expressed in Clu119 cells, so to be certain that the analogs are acting on the 5-HT2A receptor, pretreatment with ketanserin is used as a control.

In the future, more analogs should be tested to gather more information about the structure of ligands that can either activate or antagonize the 5-HT2A/$G\alpha_{q/11}$ or the 5-HT2A/JAK2/STAT3 pathways. Based on the data collected, new analogs can be created to identify analogs that can be used to study these pathways independently. These analogs can be studied in neuronal primary culture to determine the downstream effect of each pathway individually. Furthermore, these new analogs can be used in schizophrenic animal models to investigate the effect each pathway has on positive, negative, and/or cognitive symptoms and possibly if the 5-HT2A receptor has an impact on the side effects of atypical antipsychotics.

Investigating the functional selectivity of different receptors presents a new way of developing and understanding new treatments for a variety of disorders, including schizophrenia. Patients have been using atypical antipsychotics for decades with varying responses. Understanding how these drugs interact with receptors is important for improving the lives of people living with schizophrenia. The analogs tested in this dissertation and those that will be studied in the future is the first step in creating better treatment for schizophrenic patients.

References

Aaronson, D. S. and C. M. Horvath (2002). "A road map for those who don't know JAK-STAT." Science **296**(5573): 1653-1655.
Abi-Dargham, A. (2014). "Schizophrenia: overview and dopamine dysfunction." J Clin Psychiatry **75**(11): e31.
Adams, K. H., E. S. Hansen, L. H. Pinborg, S. G. Hasselbalch, C. Svarer, S. Holm, T. G. Bolwig and G. M. Knudsen (2005). "Patients with obsessive-compulsive disorder have increased 5-HT2A receptor binding in the caudate nuclei." Int J Neuropsychopharmacol **8**(3): 391-401.
Addington, J. and R. Heinssen (2012). "Prediction and prevention of psychosis in youth at clinical high risk." Annu Rev Clin Psychol **8**: 269-289.
Alonso, N., C. D. Zappia, M. Cabrera, C. A. Davio, C. Shayo, F. Monczor and N. C. Fernandez (2015). "Physiological implications of biased signaling at histamine H2 receptors." Front Pharmacol **6**: 45.
Amato, D. (2015). "Serotonin in antipsychotic drugs action." Behav Brain Res **277**: 125-135.
Arnt, J. (1998). "Pharmacological differentiation of classical and novel antipsychotics." Int Clin Psychopharmacol **13 Suppl 3**: S7-14.
Aznar, S. and S. Hervig Mel (2016). "The 5-HT2A serotonin receptor in executive function: Implications for neuropsychiatric and neurodegenerative diseases." Neurosci Biobehav Rev **64**: 63-82.
Azzi, M., P. G. Charest, S. Angers, G. Rousseau, T. Kohout, M. Bouvier and G. Pineyro (2003). "Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors." Proc Natl Acad Sci U S A **100**(20): 11406-11411.
Banes, A. K., S. M. Shaw, A. Tawfik, B. P. Patel, S. Ogbi, D. Fulton and M. B. Marrero (2005). "Activation of the JAK/STAT pathway in vascular smooth muscle by serotonin." Am J Physiol Cell Physiol **288**(4): C805-812.
Barnes, N. M. and T. Sharp (1999). "A review of central 5-HT receptors and their function." Neuropharmacology **38**(8): 1083-1152.
Barnes, T. R. and M. A. McPhillips (1998). "Novel antipsychotics, extrapyramidal side effects and tardive dyskinesia." Int Clin Psychopharmacol **13 Suppl 3**: S49-57.
Bennett, J. P., Jr., S. J. Enna, D. B. Bylund, J. C. Gillin, R. J. Wyatt and S. H. Snyder (1979). "Neurotransmitter receptors in frontal cortex of schizophrenics." Arch Gen Psychiatry **36**(9): 927-934.
Berg, K. A., W. P. Clarke, Y. Chen, B. J. Ebersole, R. D. McKay and S. Maayani (1994). "5-Hydroxytryptamine type 2A receptors regulate cyclic AMP accumulation in a neuronal cell line by protein kinase C-dependent and calcium/calmodulin-dependent mechanisms." Mol Pharmacol **45**(5): 826-836.
Berg, K. A., S. Maayani, J. Goldfarb, C. Scaramellini, P. Leff and W. P. Clarke (1998). "Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus." Mol Pharmacol **54**(1): 94-104.
Berger, M., J. A. Gray and B. L. Roth (2009). "The expanded biology of serotonin." Annu Rev Med **60**: 355-366.
Biosystems, C. C. "Hypothalamic Cell Lines." 2016.

Blaazer, A. R., P. Smid and C. G. Kruse (2008). "Structure-activity relationships of phenylalkylamines as agonist ligands for 5-HT(2A) receptors." ChemMedChem **3**(9): 1299-1309.
Buckley, P. F. and S. M. Stahl (2007). "Pharmacological treatment of negative symptoms of schizophrenia: therapeutic opportunity or cul-de-sac?" Acta Psychiatr Scand **115**(2): 93-100.
Bymaster, F. P., D. O. Calligaro, J. F. Falcone, R. D. Marsh, N. A. Moore, N. C. Tye, P. Seeman and D. T. Wong (1996). "Radioreceptor binding profile of the atypical antipsychotic olanzapine." Neuropsychopharmacology **14**(2): 87-96.
Cabungcal, J. H., D. S. Counotte, E. M. Lewis, H. A. Tejeda, P. Piantadosi, C. Pollock, G. G. Calhoon, E. M. Sullivan, E. Presgraves, J. Kil, L. E. Hong, M. Cuenod, K. Q. Do and P. O'Donnell (2014). "Juvenile antioxidant treatment prevents adult deficits in a developmental model of schizophrenia." Neuron **83**(5): 1073-1084.
Cook, E. H., Jr., K. E. Fletcher, M. Wainwright, N. Marks, S. Y. Yan and B. L. Leventhal (1994). "Primary structure of the human platelet serotonin 5-HT2A receptor: identify with frontal cortex serotonin 5-HT2A receptor." J Neurochem **63**(2): 465-469.
Costall, B. and R. J. Naylor (2004). "5-HT3 receptors." Curr Drug Targets CNS Neurol Disord **3**(1): 27-37.
Creese, I., D. R. Burt and S. H. Snyder (1976). "Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs." Science **192**(4238): 481-483.
Dai, J. X., H. L. Han, M. Tian, J. Cao, J. B. Xiu, N. N. Song, Y. Huang, T. L. Xu, Y. Q. Ding and L. Xu (2008). "Enhanced contextual fear memory in central serotonin-deficient mice." Proc Natl Acad Sci U S A **105**(33): 11981-11986.
Dai, Y., N. L. Dudek, Q. Li and N. A. Muma (2011). "Phospholipase C, Ca2+, and calmodulin signaling are required for 5-HT2A receptor-mediated transamidation of Rac1 by transglutaminase." Psychopharmacology (Berl) **213**(2-3): 403-412.
Dwivedi, Y., A. C. Mondal, H. S. Rizavi and R. R. Conley (2005). "Suicide brain is associated with decreased expression of neurotrophins." Biol Psychiatry **58**(4): 315-324.
Eison, A. S. and U. L. Mullins (1996). "Regulation of central 5-HT2A receptors: a review of in vivo studies." Behav Brain Res **73**(1-2): 177-181.
Fiorica-Howells, E., R. Hen, J. Gingrich, Z. Li and M. D. Gershon (2002). "5-HT(2A) receptors: location and functional analysis in intestines of wild-type and 5-HT(2A) knockout mice." Am J Physiol Gastrointest Liver Physiol **282**(5): G877-893.
Fullard, J. F., T. B. Halene, C. Giambartolomei, V. Haroutunian, S. Akbarian and P. Roussos (2016). "Understanding the genetic liability to schizophrenia through the neuroepigenome." Schizophr Res.
Goldberg, T. E., R. S. Goldman, K. E. Burdick, A. K. Malhotra, T. Lencz, R. C. Patel, M. G. Woerner, N. R. Schooler, J. M. Kane and D. G. Robinson (2007). "Cognitive improvement after treatment with second-generation antipsychotic medications in first-episode schizophrenia: is it a practice effect?" Arch Gen Psychiatry **64**(10): 1115-1122.
Guillet-Deniau, I., A. F. Burnol and J. Girard (1997). "Identification and localization of a skeletal muscle secrotonin 5-HT2A receptor coupled to the Jak/STAT pathway." J Biol Chem **272**(23): 14825-14829.
He, L., J. Fong, M. von Zastrow and J. L. Whistler (2002). "Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization." Cell **108**(2): 271-282.

Hoyer, D., D. E. Clarke, J. R. Fozard, P. R. Hartig, G. R. Martin, E. J. Mylecharane, P. R. Saxena and P. P. Humphrey (1994). "International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin)." Pharmacol Rev **46**(2): 157-203.
Ji, K., J. Miyauchi and S. E. Tsirka (2013). "Microglia: an active player in the regulation of synaptic activity." Neural Plast **2013**: 627325.
Joyce, E. M. and J. P. Roiser (2007). "Cognitive heterogeneity in schizophrenia." Curr Opin Psychiatry **20**(3): 268-272.
Kantrowitz, J. T. and D. C. Javitt (2010). "N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: the final common pathway on the road to schizophrenia?" Brain Res Bull **83**(3-4): 108-121.
Kasper, S., A. Hale, J. M. Azorin and H. J. Moller (1999). "Benefit-risk evaluation of olanzapine, risperidone and sertindole in the treatment of schizophrenia." Eur Arch Psychiatry Clin Neurosci **249 Suppl 2**: II1-II14.
Kenakin, T. (1995). "Agonist-receptor efficacy. II. Agonist trafficking of receptor signals." Trends Pharmacol Sci **16**(7): 232-238.
Kenakin, T. (2001). "Inverse, protean, and ligand-selective agonism: matters of receptor conformation." FASEB J **15**(3): 598-611.
Kenakin, T. (2011). "Functional selectivity and biased receptor signaling." J Pharmacol Exp Ther **336**(2): 296-302.
Khandaker, G. M., J. Zimbron, G. Lewis and P. B. Jones (2013). "Prenatal maternal infection, neurodevelopment and adult schizophrenia: a systematic review of population-based studies." Psychol Med **43**(2): 239-257.
Kunugi, H., T. Urushibara, R. M. Murray, S. Nanko and T. Hirose (2003). "Prenatal underdevelopment and schizophrenia: a case report of monozygotic twins." Psychiatry Clin Neurosci **57**(3): 271-274.
Kurrasch-Orbaugh, D. M., J. C. Parrish, V. J. Watts and D. E. Nichols (2003). "A complex signaling cascade links the serotonin2A receptor to phospholipase A2 activation: the involvement of MAP kinases." J Neurochem **86**(4): 980-991.
Kurrasch-Orbaugh, D. M., V. J. Watts, E. L. Barker and D. E. Nichols (2003). "Serotonin 5-hydroxytryptamine 2A receptor-coupled phospholipase C and phospholipase A2 signaling pathways have different receptor reserves." J Pharmacol Exp Ther **304**(1): 229-237.
Leysen, J. E. (2004). "5-HT2 receptors." Curr Drug Targets CNS Neurol Disord **3**(1): 11-26.
Leysen, J. E., D. de Chaffoy de Courcelles, F. De Clerck, C. J. Niemegeers and J. M. Van Nueten (1984). "Serotonin-S2 receptor binding sites and functional correlates." Neuropharmacology **23**(12B): 1493-1501.
Lieberman, J. A., D. Perkins, A. Belger, M. Chakos, F. Jarskog, K. Boteva and J. Gilmore (2001). "The early stages of schizophrenia: speculations on pathogenesis, pathophysiology, and therapeutic approaches." Biol Psychiatry **50**(11): 884-897.
Llorca, P. M., I. Chereau, F. J. Bayle and C. Lancon (2002). "Tardive dyskinesias and antipsychotics: a review." Eur Psychiatry **17**(3): 129-138.
Marti-Solano, M., A. Iglesias, G. de Fabritiis, F. Sanz, J. Brea, M. I. Loza, M. Pastor and J. Selent (2015). "Detection of new biased agonists for the serotonin 5-HT2A receptor: modeling and experimental validation." Mol Pharmacol **87**(4): 740-746.
Matsumoto, I., Y. Inoue, T. Iwazaki, G. Pavey and B. Dean (2005). "5-HT2A and muscarinic receptors in schizophrenia: a postmortem study." Neurosci Lett **379**(3): 164-168.

Meltzer, H. Y. (1999). "The role of serotonin in antipsychotic drug action." Neuropsychopharmacology **21**(2 Suppl): 106S-115S.
Meltzer, H. Y., Z. Li, Y. Kaneda and J. Ichikawa (2003). "Serotonin receptors: their key role in drugs to treat schizophrenia." Prog Neuropsychopharmacol Biol Psychiatry **27**(7): 1159-1172.
Miller, K. J., C. L. Mariano and W. R. Cruz (1997). "Serotonin 5HT2A receptor activation inhibits inducible nitric oxide synthase activity in C6 glioma cells." Life Sci **61**(18): 1819-1827.
Moghaddam, B. and D. Javitt (2012). "From revolution to evolution: the glutamate hypothesis of schizophrenia and its implication for treatment." Neuropsychopharmacology **37**(1): 4-15.
Mottola, D. M., S. Laiter, V. J. Watts, A. Tropsha, S. D. Wyrick, D. E. Nichols and R. B. Mailman (1996). "Conformational analysis of D1 dopamine receptor agonists: pharmacophore assessment and receptor mapping." J Med Chem **39**(1): 285-296.
Moya, P. R., K. A. Berg, M. A. Gutierrez-Hernandez, P. Saez-Briones, M. Reyes-Parada, B. K. Cassels and W. P. Clarke (2007). "Functional selectivity of hallucinogenic phenethylamine and phenylisopropylamine derivatives at human 5-hydroxytryptamine (5-HT)2A and 5-HT2C receptors." J Pharmacol Exp Ther **321**(3): 1054-1061.
Muma, N. A. and Z. Mi (2015). "Serotonylation and Transamidation of Other Monoamines." ACS Chem Neurosci **6**(7): 961-969.
Muma, N. A., R. K. Singh, M. S. Vercillo, D. N. D'Souza, B. Zemaitaitis, F. Garcia, K. J. Damjanoska, Y. Zhang, G. Battaglia and L. D. Van de Kar (2007). "Chronic olanzapine activates the Stat3 signal transduction pathway and alters expression of components of the 5-HT2A receptor signaling system in rat frontal cortex." Neuropharmacology **53**(4): 552-562.
Muschamp, J. W., M. J. Regina, E. M. Hull, J. C. Winter and R. A. Rabin (2004). "Lysergic acid diethylamide and [-]-2,5-dimethoxy-4-methylamphetamine increase extracellular glutamate in rat prefrontal cortex." Brain Res **1023**(1): 134-140.
Nicolas, C. S., M. Amici, Z. A. Bortolotto, A. Doherty, Z. Csaba, A. Fafouri, P. Dournaud, P. Gressens, G. L. Collingridge and S. Peineau (2013). "The role of JAK-STAT signaling within the CNS." JAKSTAT **2**(1): e22925.
NIMH. (2016). "Schizophrenia." 2016, from http://www.nimh.nih.gov/health/publications/schizophrenia-booklet-12-2015/index.shtml#pub3.
Owen, M. J., A. Sawa and P. B. Mortensen (2016). "Schizophrenia." Lancet **388**(10039): 86-97.
Paredes, R. M., J. C. Etzler, L. T. Watts, W. Zheng and J. D. Lechleiter (2008). "Chemical calcium indicators." Methods **46**(3): 143-151.
Polter, A. M. and X. Li (2010). "5-HT1A receptor-regulated signal transduction pathways in brain." Cell Signal **22**(10): 1406-1412.
Rankovic, Z., T. F. Brust and L. M. Bohn (2016). "Biased agonism: An emerging paradigm in GPCR drug discovery." Bioorg Med Chem Lett **26**(2): 241-250.
Raote, I., A. Bhattacharya and M. M. Panicker (2007). Serotonin 2A (5-HT2A) Receptor Function: Ligand-Dependent Mechanisms and Pathways. Serotonin Receptors in Neurobiology. A. Chattopadhyay. Boca Raton (FL).
Raote, I., S. Bhattacharyya and M. M. Panicker (2013). "Functional selectivity in serotonin receptor 2A (5-HT2A) endocytosis, recycling, and phosphorylation." Mol Pharmacol **83**(1): 42-50.
Rawlings, J. S., K. M. Rosler and D. A. Harrison (2004). "The JAK/STAT signaling pathway." J Cell Sci **117**(Pt 8): 1281-1283.
Sawa, A. and L. J. Seidman (2014). "Is prophylactic psychiatry around the corner? combating adolescent oxidative stress for adult psychosis and schizophrenia." Neuron **83**(5): 991-993.

Schafer, D. P., E. K. Lehrman, A. G. Kautzman, R. Koyama, A. R. Mardinly, R. Yamasaki, R. M. Ransohoff, M. E. Greenberg, B. A. Barres and B. Stevens (2012). "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner." Neuron **74**(4): 691-705.
Seeman, P. (2010). "Dopamine D2 receptors as treatment targets in schizophrenia." Clin Schizophr Relat Psychoses **4**(1): 56-73.
Shen, W. W. (1999). "A history of antipsychotic drug development." Compr Psychiatry **40**(6): 407-414.
Singh, R. K., Y. Dai, J. L. Staudinger and N. A. Muma (2009). "Activation of the JAK-STAT pathway is necessary for desensitization of 5-HT2A receptor-stimulated phospholipase C signalling by olanzapine, clozapine and MDL 100907." Int J Neuropsychopharmacol **12**(5): 651-665.
Singh, R. K., J. Shi, B. W. Zemaitaitis and N. A. Muma (2007). "Olanzapine increases RGS7 protein expression via stimulation of the Janus tyrosine kinase-signal transducer and activator of transcription signaling cascade." J Pharmacol Exp Ther **322**(1): 133-140.
Ucok, A. and W. Gaebel (2008). "Side effects of atypical antipsychotics: a brief overview." World Psychiatry **7**(1): 58-62.
Unsworth, C. D. and P. B. Molinoff (1992). "Regulation of the 5-hydroxytryptamine1B receptor in opossum kidney cells after exposure to agonists." Mol Pharmacol **42**(3): 464-470.
Urban, J. D., W. P. Clarke, M. von Zastrow, D. E. Nichols, B. Kobilka, H. Weinstein, J. A. Javitch, B. L. Roth, A. Christopoulos, P. M. Sexton, K. J. Miller, M. Spedding and R. B. Mailman (2007). "Functional selectivity and classical concepts of quantitative pharmacology." J Pharmacol Exp Ther **320**(1): 1-13.
Vaidya, V. A., G. J. Marek, G. K. Aghajanian and R. S. Duman (1997). "5-HT2A receptor-mediated regulation of brain-derived neurotrophic factor mRNA in the hippocampus and the neocortex." J Neurosci **17**(8): 2785-2795.
Van de Kar, L. D., A. Javed, Y. Zhang, F. Serres, D. K. Raap and T. S. Gray (2001). "5-HT2A receptors stimulate ACTH, corticosterone, oxytocin, renin, and prolactin release and activate hypothalamic CRF and oxytocin-expressing cells." J Neurosci **21**(10): 3572-3579.
Walther, D. J., J. U. Peter, S. Bashammakh, H. Hortnagl, M. Voits, H. Fink and M. Bader (2003). "Synthesis of serotonin by a second tryptophan hydroxylase isoform." Science **299**(5603): 76.
Wei, H., S. Ahn, S. K. Shenoy, S. S. Karnik, L. Hunyady, L. M. Luttrell and R. J. Lefkowitz (2003). "Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2." Proc Natl Acad Sci U S A **100**(19): 10782-10787.
Whalen, E. J., S. Rajagopal and R. J. Lefkowitz (2011). "Therapeutic potential of beta-arrestin- and G protein-biased agonists." Trends Mol Med **17**(3): 126-139.
Willins, D. L., S. A. Berry, L. Alsayegh, J. R. Backstrom, E. Sanders-Bush, L. Friedman and B. L. Roth (1999). "Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors in vitro and in vivo." Neuroscience **91**(2): 599-606.
Yu, Y., L. Zhang, X. Yin, H. Sun, G. R. Uhl and J. B. Wang (1997). "Mu opioid receptor phosphorylation, desensitization, and ligand efficacy." J Biol Chem **272**(46): 28869-28874.