Novel Role of the JAK-STAT Pathway in Mediating the Effects of Atypical Antipsychotics on 5-HT_{2A} Receptor Signaling

By

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University of Kansas, 2008

Submitted to the Department of Pharmacology and Toxicology and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirement for the degree of Doctor of Philosophy

Dissertation Committee:

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ABSTRACT

The therapeutic benefits of atypical antipsychotics are proposed to be mediated by antagonism and subsequent desensitization of 5-HT_{2A} receptor signaling; however, the mechanisms underlying this desensitization response are not yet understood. We hypothesize that the desensitization of 5-HT_{2A} receptors induced by atypical antipsychotics is dependent on activation of the JAK-STAT pathway. To test this hypothesis, we used a cell line, A1A1v cells, that natively expresses 5-HT_{2A} receptor signaling system, and further confirmed the findings in rats. In A1A1v cells, we confirmed that treatment with both olanzapine and clozapine desensitizes 5-HT_{2A} receptor signaling. Furthermore, olanzapine treatment also increased RGS7 mRNA and protein levels which were dependent on activation of JAK-STAT pathway. Similar results were found with MDL100907, a specific $5HT_{2A}$ receptor antagonist; RGS7 protein and mRNA levels were increased along with activation of the JAK-STAT pathway, suggesting that antagonism of 5-HT_{2A} receptors is sufficient to induce these changes. In addition, we also found an increase in STAT3 binding to the putative RGS7 promoter region with olanzapine treatment suggesting that the increase in RGS7 expression could be directly mediated by the JAK-STAT pathway. An increase in RGS protein could mediate desensitization of 5-HT_{2A} receptor signaling by its GAP activity. Lastly, inhibition of the JAK-STAT pathway significantly attenuated olanzapine-induced desensitization of 5-HT_{2A} receptor signaling in A1A1v

cells. Similar findings were also observed in rats treated with olanzapine for 7 days. We found a decrease in 5-HT_{2A} receptor-stimulated PLC activity in the frontal cortex which was dependent on activation of JAK-STAT pathway. Consistent with the cell culture data, the olanzapine-induced increase in RGS7 proteins and mRNA levels were dependent on activation of the JAK-STAT pathway. Olanzapine treatment significantly reduced plasma levels of oxytocin, adrenocorticotrophic hormone (ACTH), and corticosterone. Surprisingly, 5-HT_{2A} receptor-stimulated oxytocin and corticosterone levels were also decreased in a dose-dependent manner by the JAK inhibitor whereas ACTH levels were not altered. Further studies are needed to investigate the role of the JAK-STAT pathway in the regulation of hormone levels. Taken together, these results from experiments in cells in culture and in rats suggest that increases in RGS7 expression via increased activation of the JAK-STAT pathway are necessary for antipsychotic-induced desensitization of 5-HT_{2A} receptor signaling.

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DEDICATION

For my parents

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (Serotonin)
ACTH	Adenocorticopin hormone
CaM-Kinase	Ca^{2+}/calmodulin-dependent protein
CRF1	Corticotropin-releasing factor
DAG	Diacylglycerol
DOI	(-)-1-(2,5-Dimethoxy-4-Iodophenyl)-2-aminopropane HCl
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
EDTA	Ethylene diamine tetraacetic acid
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
EGFR	Epidermal growth factor receptor
EPO	Erythropoietin
EPS	Extrapyramidal symptoms
ERK	Extracellular signal-regulated kinase
GABA	γ-aminobutyric acid
GAP	GTPase accelerating protein
GFP	Green fluorescent protein

GAPDH	Glyceraldehyde3-phosphate dehydrogenase
GGL	G protein -like domain
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GSK	Glycogen synthase kinase
HB-EFG	Heparin-bound epidermal growth factor
HEK-293	Human Embryonic Kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic pituitary axis
IFN-α	Interferon
IL	Interleukins
IP3	Inositol Phosphate
JAK	Janus kinase
LSD	Lysergic Acid Diathylamide
MAO	Monoamine oxidase
MDL 100,907	[R-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-Fluorophenylethyl)]-4-piperidinem ethanol]
MEK	Mitogen-activated protein kinase/ extracell--ular signal-regulated kinase

MS	Multiple sclerosis
NCSC	Nomenclature Committee of the
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
	of transcription
PC12	Pheochromocytoma of the rat adrenal medulla
PCPA	Parachorophenylalanine
PCR	Polymerase chain reaction
PET	Positron emission tomography
PIAS	Protein inhibitor of activated STAT
PKA	Protein kinase A
PKC	Protein Kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PTZ	Pentylenetetrazole
RGS	Regulator of G-protein signaling
RTK	Receptor tyrosine kinase
SAVIC	sheep aortic valve interstitial cells
SCID	Severe Combined Immunodeficiency
	Serotonin Club

SHP	Src-Homology-2-Domain-Containing Phosphatases
SOCS	Suppressor of cytokine signaling
SSRI	Selective Serotonin Reuptake Inhibitor
STAT	Signal transducer and activator
TACE	Tumor necrosis factor-activating enzyme
TAD	The transactivation domain
VSMC	Vascular smooth muscle cells
VTA	Ventral tegmental area

CHAPTER I

INTRODUCTION

Atypical antipsychotics are widely prescribed for the treatment of schizophrenia. They are classified as atypical because of their ability to achieve antipsychotic effects with lower rates of extrapyramidal side effects compared with first generation antipsychotics such as haloperidol. In addition, selected atypical antipsychotics also improve certain aspects of cognitive function in schizophrenic patients, whereas typical antipsychotics may worsen cognition (Meltzer, 1999). Although, atypical antipsychotics have a diverse receptor binding profile, 5-HT-receptor-based mechanisms have been postulated to play a critical role in the action of the atypical antipsychotic drugs (Willins, et al., 1999). However, the process by which these drug-receptor interactions translate into long-term cellular adaptive changes resulting in antipsychotic efficacy is unknown. Atypical antipsychotic drugs bind with high affinity to 5-HT_{2A} receptors and desensitize 5-HT_{2A} receptor signaling (Deutch, et al., 1991; Meltzer and Nash, 1991; Seeger, et al., 1995). Desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics is reported to be associated with down-regulation and internalization (Willins, et al., 1999). The 5-HT_{2A} receptor subtype has been implicated in various psychiatric disorders including depression, anxiety, and schizophrenia (Glennon, et al., 1984). Atypical antipsychotics as well as a specific 5-HT_{2A} receptor antagonist, MDL 100,907, desensitize 5-HT_{2A}-mediated responses

(Willins, et al., 1999). However, the molecular mechanisms involved in antagonist-induced desensitization of 5-HT_{2A} receptor signaling are not well understood. By understanding the molecular mechanisms underlying the effects of olanzapine and other atypical antipsychotics, we hope to gain insight into targets for therapeutic treatment of psychiatric disorders.

The Janus tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT) signaling cascade has been reported to couple with 5-HT_{2A} receptors in skeletal muscles and vascular smooth muscle cells (Guillet-Deniau, et al., 1997; Banes, et al., 2005). G protein-coupled receptor agonists, thrombin and angiotensin II, have previously been shown to activate the JAK-STAT signaling cascade (Bhat, et al., 1994). JAK-STAT could be one of the possible signaling pathways involved in mediating olanzapine-induced receptor desensitization. JAKs are a small family of cytoplasmic tyrosine kinases initially identified as a mediator of cytokine receptor signaling (Ihle, 1995). Agonist stimulation of cytokine receptors causes phosphorylation of JAK, which in turn phosphorylates tyrosine residues on the receptor cytoplasmic tail, facilitating activation of specific STATs. Tyrosine phosphorylated STAT, then undergoes dimerization and translocates to the nucleus, where it binds to target DNA sequences (Darnell, Jr., 1998). Alterations in proximal components of the 5-HT_{2A} receptor signaling system could mediate desensitization in response to increased activity of intracellular cascades such as JAK-STAT. 5-HT_{2A} receptors are classically linked to the

$G_{\alpha q}$ protein family (Ivins and Molinoff, 1990). Activation of $G_{\alpha q}$ stimulates phospholipase C activity, which subsequently promotes the release of diacylglycerol and inositol triphosphate, which in turn stimulate protein kinase C activity and calcium release (Berg, et al., 2001). It has been extensively reported that increased expression of regulator of G protein signaling (RGS) proteins cause desensitization of several G protein-associated receptor systems (Koelle and Horvitz, 1996). RGS proteins reduce the duration of signaling of many G protein-coupled receptors by their action as GTPases, accelerating the hydrolysis of GTP-bound G_{α} proteins or by blocking the interaction of G_{α} with its target proteins through a not well understood process known as effector antagonism (Bramow-Newerly, et al., 2006). Expression of RGS7 protein in rat frontal cortex is well documented (Zhang and Simonds, 2000;Krumins, et al., 2004) and decreased 5-HT_{2A} receptor-mediated signaling via direct interaction of RGS7 protein with $G_{\alpha q}$ has been widely characterized in different systems (DiBello, et al., 1998;Ghavami, et al., 2004).

In addition, atypical antipsychotics are reported to reduce levels of ACTH and cortisol in schizophrenic patients (Cohrs, et al., 2006; Scheepers, et al., 2001; Hatzimanolis, et al., 1998; Meltzer, 1989; Markianos, et al., 1999). Although, monoaminergic mechanisms including serotonin and dopamine are known to play an important role in the regulation of ACTH and cortisol secretion (Wilcox, et al., 1975; Fuller and Snoddy, 1984;Tuomisto and Mannisto, 1985; Contesse, et al., 2000), the attenuation of cortisol secretion,

after subchronic administration of olanzapine and clozapine to schizophrenic patients, has been attributed to 5-HT receptor blockade (Scheepers, et al., 2001; Hatzimanolis, et al., 1998; Markianos, et al., 1999). Furthermore, 5-HT_{2A} receptors in the hypothalamic paraventricular nucleus have been shown to mediate the neuroendocrine responses to a peripheral injection of DOI; intraparaventricular and peripheral injections of the selective 5-HT_{2A} receptor antagonist MDL 100,907 dose dependently inhibit the DOI-induced increases in hormone secretions (Zhang, et al., 2002). These data suggest that decreases in hormone secretion by atypical antipsychotics may be mediated by 5-HT_{2A} receptors and that plasma hormone levels can be monitored as an index of the function of 5-HT_{2A} receptor signaling in the hypothalamic paraventricular nucleus.

Central hypothesis

The desensitization of 5-HT_{2A} receptor signaling induced by chronic antagonist treatment is dependent on JAK-STAT signaling and subsequent changes in gene expression, especially an increase in RGS7 protein. Three different specific aims are proposed to test this hypothesis.

The **first specific aim** of this dissertation is to determine the underlying mechanisms of olanzapine-induced desensitization of 5-HT_{2A} receptor signaling in A1A1v cells. Experiments are proposed to test whether olanzapine-induced desensitization is mediated by alterations in signaling components of 5-HT_{2A} receptor such as RGS proteins and receptor levels. In

addition, we will determine the possible role of activation of the JAK-STAT pathway on the desensitization response and whether inhibition of this pathway could reduce the desensitization response caused by olanzapine. The **second specific aim** is to determine the selectivity of atypical antipsychotics on desensitization of 5-HT_{2A} receptor signaling and regulation of RGS7 protein expression. Specifically, whether clozapine-induced desensitization is mediated by activation of JAK-STAT pathway and RGS7 expression. Furthermore, we will determine if antagonism of 5-HT_{2A} receptors is sufficient for desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics. The **third specific aim** is to determine the involvement of the JAK-STAT signaling in the olanzapine-induced desensitization of 5-HT_{2A} receptor signaling *in vivo*. Specifically, in this aim we will investigate the role of the JAK-STAT pathway in the frontal cortex (5-HT_{2A}-mediated-PLC activity) and the paraventricular nucleus (hormone levels) of rats in response to chronic treatment with olanzapine.

CHAPTER II

REVIEW OF RELATED LITERATURE

Serotonin

Discovery:

The discovery of serotonin (5-HT) can be traced back to the late 1940's when it was reported that the serum of clotted blood contained a factor capable of causing vasoconstriction. Eventually, the indolamine 5-HT was discovered by Rapport et al. (1948) and was found to have vasoconstriction and blood clotting properties (Rapport, et al., 1948). Independently, Erspamer and Ottolenghi (1950) had discovered a factor (enteramine) in gut mucosa that was later shown to be identical to 5-HT (Erspamer and Ottolenghi, 1950). Twarog and Page (1953) finally discovered that 5-HT was present in brain and this finding led others to establish 5-HT as a neurotransmitter (Twarog and Page, 1953). 5-HT is found in three major areas of the body; the intestinal wall (isolated as enteramine by Dr. Erspamer, where it causes increased gastrointestinal mobility); blood vessels (isolated and named as serotonin by Dr. Page's laboratory, where it causes vasoconstriction); and the central nervous system (CNS) (isolated by Dr. Twarog).

Brain Distribution of 5-HT:

The anatomical distribution of 5-HT neurons in the brain was first studied by fluorescence histochemical detection of the indoleamine by Dahlstrom and Fuxe (Dahlstrom and Fuxe, 1964). In 1964, they described 5-HT neurons as a relatively small population of morphologically diverse neurons whose cell bodies are present largely within the brainstem raphe nuclei and particular regions of the reticular formation. Raphe clusters of 5-HT neurons are found rostrally from the level of the interpeduncular nucleus in the midbrain to the level of the pyramidal decussation in the medulla.

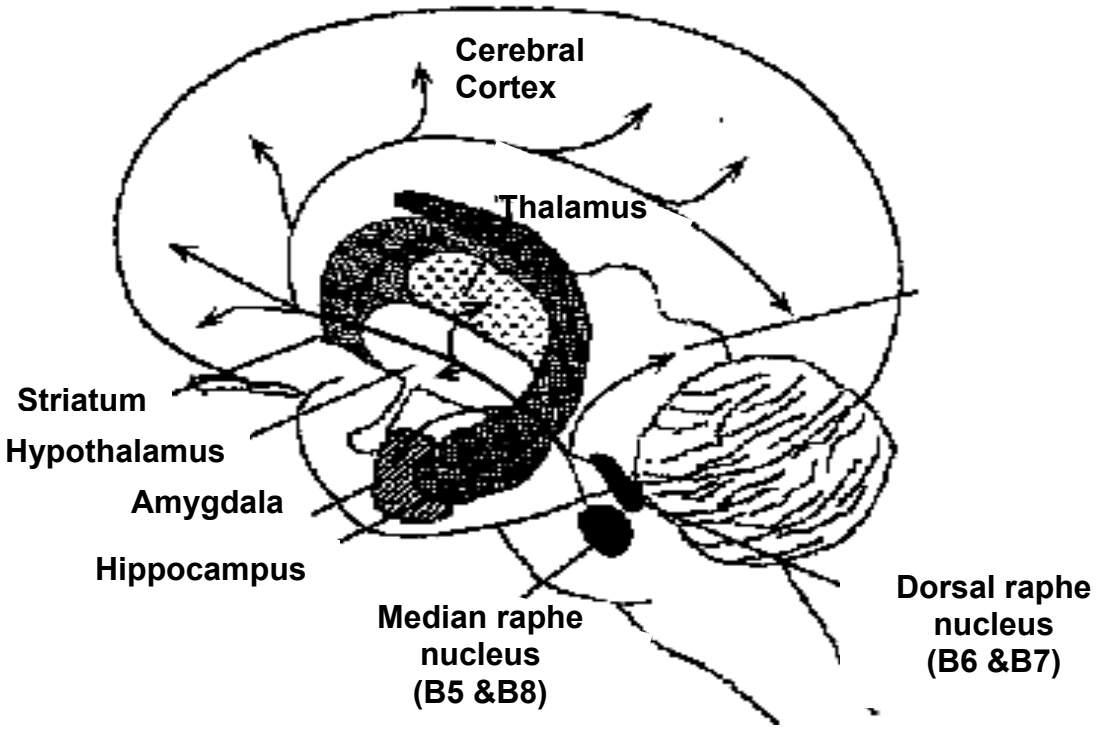


Figure 1: Serotonergic projections in the human brain. Includes neuronal projections (arrow) and serotonergic nuclei in the brain stem (ovals). (Picture adapted from (Bear and Abraham, 1996).

Although there are only about 20,000 serotonergic neurons in the rat brain (~300K in humans), the extensive axonal projection system arising from these neurons bears a tremendous number of collateral branches so that the 5-HT system densely innervates nearly all regions of the CNS (Dahlstrom and Fuxe, 1964). The midline raphe nuclei consist of the caudal linear nucleus (CLi, B8), the dorsal raphe nucleus (DR, B6, B7), the median raphe nucleus (MnR, B5, B8), raphe magnus nucleus (RMg, B3), raphe pallidus nucleus (RPa, B1), and the raphe obscurus nucleus (ROb, B2) (Dahlstrom and Fuxe, 1964; Dahlstrom and Fuxe, 1964; Fuxe and Gunne, 1964; Dahlstroem, et al., 1964). Innervations of the ascending structures by serotonergic neurons are primarily derived from the dorsal raphe and median raphe (Figure 1), and innervate the cortex, limbic system, basal ganglia and hypothalamus. The cortex, striatum, and globus pallidus are predominantly innervated by dorsal raphe nucleus; whereas median raphe predominantly innervates medial septum and dorsal hippocampus. In addition, the ventral hippocampal region is innervated by both the dorsal raphe nucleus and the median raphe nucleus (McQuade and Sharp, 1997).

Initial 5-HT Studies:

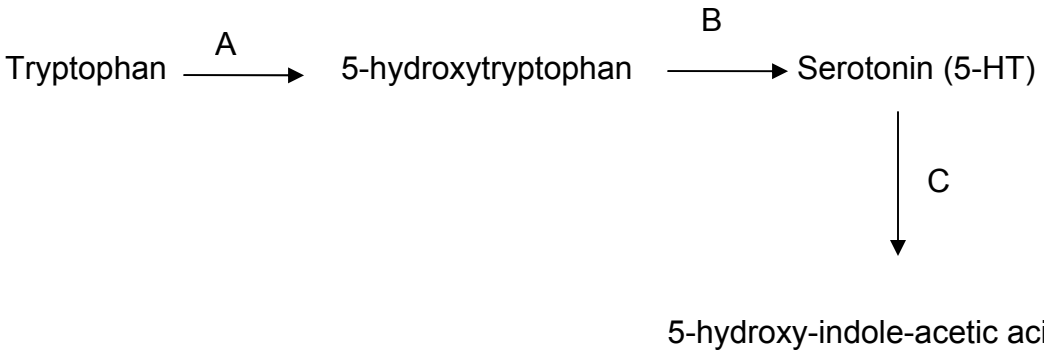
After the discovery of 5-HT, researchers primarily focused on defining the pathways for synthesis and degradation of 5-HT. One of the first clinical applications of this new found understanding was the use of 5-HT synthesis inhibitor, parachorophenylalanine (PCPA), as a treatment to reduce excessive 5-HT secretions from carcinoid tumors (Carrillo and Aviado, 1969). However, a great deal of interest soon focused on 5-HT because of its presumed involvement in the major psychoses (Heym, et al., 1984; Himwich and Costa, 1960; Consbruch and Faust, 1960; Sourkes, 1956; Pelicier, 1964). The development of monoamine oxidase inhibitors and their effectiveness in the treatment of depression provided an early indication of the importance of 5-HT in these psychiatric disorders.

5-HT is involved in diverse physiological and behavioral functions such as aggression, appetite, cognition, emesis, endocrine function, gastrointestinal function, sex, sleep, and vascular function (Reynolds, III, et al., 1995; Heninger, 1997). 5-HT has been also implicated in the etiology of many disease states and particularly important in mental illness such as depression, anxiety, obsessive compulsive disorder, panic disorder, migraine, and eating disorders (Sjoerdsma and Palfreyman, 1990). Indeed, many treatments currently used for these disease states are thought to act by modulating serotonergic tone, highlighting the need of developing more

selective ligands that could lead to novel therapies with increased efficacy and decreased side effects.

Biosynthesis and Metabolism of 5-HT:

Considering the important and diverse role of 5-HT in the brain, one would assume that most of the 5-HT in the body would be present or synthesized in the brain. However, only 1-2% of total body 5-HT is synthesized in brain and is sufficient to maintain normal brain function. Most of the 5-HT, almost 95%, is actually found in gastrointestinal tract (GI) (90% in enterochromaffin cells and 5% in enteric neurons). Although a large amount of 5-HT is present in the periphery, it cannot cross the blood brain barrier therefore, it is synthesized in the brain under normal conditions (Sharma and Dey, 1986; Sharma, et al., 1990). Plasma L-tryptophan, which is a primary substrate for 5-HT synthesis, is derived mainly from diet and is actively transported into the brain. 5-HT can be synthesized both in the neuronal cell bodies and nerve terminals (Wilson and Marsden, 1996). The synthesis of 5-HT is described in figure 2 with tryptophan hydroxylase (A) being rate limiting enzyme. Once released from neurons, the action of 5-HT is mainly terminated by re-uptake in neurons, where it is either restored in the vesicles or metabolized by monoamine oxidase (MAO).



A. Tryptophan hydroxylase
B. Amino acid decarboxylase
C. Monoamine oxidase/ Aldehyde dehydrogenase

Figure 2: 5-HT synthesis pathway: Schematic representation of the 5-HT synthesis pathway with tryptophan as starting material. Tryptophan hydroxylase is the rate limiting enzyme.

Discovery of 5-HT Receptors:

Neurotransmitters produce their effects as a consequence of interaction with appropriate receptors. Gaddum and Picarelli (1957) were the first to suggest the existence of more than one type of 5-HT receptor (Gaddum and Picarelli, 1957). Subsequently pharmacological, neurophysiological, and other techniques have provided evidence that 5-HT acts at pre-synaptic and post-synaptic sites and could be excitatory or

inhibitory in its action. However, more definitive evidence of 5-HT receptor heterogeneity began to emerge from early 1980's.

There is now molecular and functional evidence for the existence of 16 different subtypes of 5-HT receptors (Hoyer, et al., 2002). Indeed, the multiplicity of 5-HT receptor subtypes, both within and among species, has exceeded most of the predictions that could have been made on the basis of pharmacological data in the late 1980s. While the discovery of 5-HT receptor subtypes advanced rapidly in the late 1980s and early 1990s, the number and specificity of ligands for 5-HT receptor subtypes has lagged behind. The existence of so many 5-HT receptors appears to be a result of more than 750 million years of molecular evolution. Advances in molecular biological analysis of 5-HT receptors have made the classification procedure more logical (Lucas and Hen, 1995).

Classification of 5-HT Receptors:

Bradley et al. (1986) initiated a classification scheme for the subtypes of 5-HT receptors (Bradley, et al., 1986). His rationale was based on binding studies of selective pharmacological agents like methiothepin, methylsergide and 5-carboxymidotryptamine. The Nomenclature Committee of the Serotonin Club (NCSC) updated and modified this list in 1993 to include recently cloned receptors (Humphrey, et al., 1993).

The current system of classification depicted below is inclusive of new information obtained with both recombinant and native receptors to align the nomenclature with the human genome and avoid species differences (Hartig, et al., 1996; Hoyer, et al., 1994). Currently, seven families of 5-HT receptors are recognized including 16 subtypes (multiple isoforms)(Hoyer, et al., 2002).

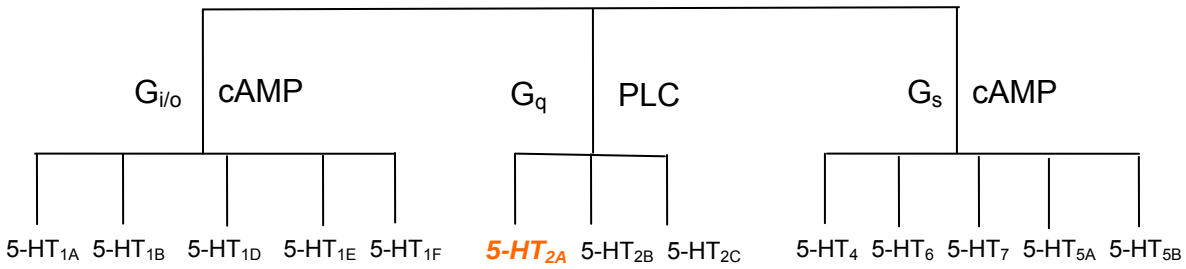


Figure 3: 5-HT receptors with effector enzymes: Schematic representation of the 5-HT receptor family based on protein sequence and structural homology (Barnes and Sharp, 1999;Hoyer, et al., 2002)

Structurally, all 5-HT receptors, except the $5HT_3$ receptor (Maricq, et al., 1991) which is a ligand gated ion channel, related to NMDA, GABA, and nicotinic receptor, belong to G-protein super-family and contain the seven transmembrane regions. At least five 5-HT_1 receptor subtypes have been recognized- 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. These receptors are negatively linked to adenyl cyclase via the G_i family of G proteins with an

overall sequence homology of about 40%. 5-HT_4 - 5-HT_7 are positively linked to adenyl cyclase via G_s. The central focus of this dissertation is on 5-HT_{2A} subclass of 5-HT receptors, this receptor subtype will be described in more detail.

5-HT_2 Receptor Subfamily:

On the basis of functional studies with agonists and antagonists, ligand binding affinities, molecular structure and intracellular transduction mechanisms, the 5-HT_2 receptor family comprises three specific subtypes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. All three are positively couple to the effector enzyme phospholipase C (PLC) via G_q and display an overall sequence homology of approx 60% (Boess and Martin, 1994; Martin and Humphrey, 1994). Activation of these receptors results in PLC-mediated increases in inositol phosphate (IP3) and increases in intracellular Ca^{++} levels (Berridge, 1982; Conn and Sanders-Bush, 1984). Ullmer et al. reported that stimulation of 5-HT_{2B} receptors causes intracellular calcium release via a mechanism independent of the phosphatidylinositol hydrolysis (Ullmer, et al., 1996).

5-HT_{2A} Receptors:

The human 5-HT_{2A} receptor gene is located on chromosome 13q14–q21 (Sparkes, et al., 1991) consists of three exons separated by two introns, and spans over 20 kb (Liu, et al., 1991). 5-HT_{2A} receptor subtypes have been

identified in both the CNS and the periphery. In periphery, they are located on vascular, bronchial, and urinary smooth muscle and on platelets. Their function includes vaso- and broncho-constriction and platelet aggregation. 5-HT_{2A} receptors are expressed widely throughout the CNS. They are expressed near most of the serotoninergic terminal rich areas, including neocortex (mainly prefrontal, parietal, and somatosensory cortex) and olfactory tubercle. There are high concentrations of this receptor on the apical dendrites of the pyramidal cells in layer V of the cortex suggested to modulate cognitive processes (Pazos, et al., 1985; Pompeiano, et al., 1994). In addition, 5-HT_{2A} receptors are predominantly located in the neocortex, with a large density in the prefrontal cortex, which suggests its involvement in higher brain functions, such as working memory (Williams, et al., 2002). In the rat, postsynaptic 5-HT_{2A} receptors in medial prefrontal cortex control the activity of the serotonergic system through changes in the activity of pyramidal neurons projecting to the dorsal raphe nucleus. Stimulation of central 5-HT_{2A} receptors in rats causes head twitches and is suggested to mediate the effects of hallucinogens such as lysergic acid diethylamide (LSD) in humans (Darmani and Gerdes, 1995). Abnormalities in 5-HT receptors especially 5-HT_{2A} receptor-mediated signal transduction systems have been widely reported in various disorders like mood disorders (Ressler and Nemeroff, 2000; Akin, et al., 2004), and specifically depression (Rosel, et al., 2000), and bulimia nervosa (binge eating and inappropriate compensatory behaviors to avoid

weight gain) (Goethals, et al., 2004; Walsh and Devlin, 1998). One of the more interesting and recent findings on the 5-HT_{2A} receptor is that it plays a very important role in schizophrenia. Although, the role of 5-HT_{2A} receptors in the etiology of schizophrenia is still debatable, there is complete agreement regarding 5-HT_{2A} receptors as an important target of atypical antipsychotics. The next section will discuss the progress made in over many decades of research on 5-HT_{2A} receptors in various CNS disorders.

5-HT_{2A} Receptors and CNS Disorders

5-HT_{2A} receptors have been implicated in numerous CNS disorders including depression, anxiety, migraine, psychosis and schizophrenia, sleep disorders, and hypertension. In addition to other monoamine neurotransmitter receptors, 5-HT_{2A} receptors serve as a major therapeutic target of antidepressant and antipsychotic drugs. The next section will highlight the role of 5-HT_{2A} receptors in depression and schizophrenia.

Depression and 5-HT_{2A} receptors:

Depression is one of the most common psychological problems. Each year over 17 million American adults experience a period of clinical depression. A decrease in the synaptic levels of monoamines (5-HT, norepinephrine, and epinephrine) in the CNS may be an underlying cause of this disorder (Delgado, 2000). With the success of selective serotonin

reuptake inhibitor (SSRIs), such as fluoxetine (Prozac), the 5-HT system became the focus of a large body of literature implicating 5-HT neurotransmitter disturbances in the pathophysiology of depression. Of the 16 different classes of 5-HT receptors identified to date, the 5-HT_{2A} and 5-HT_{1A} receptors have been most extensively investigated in psychotic disorders. Using positron emission tomography (PET) and a selective 5-HT_{2A} receptor radioligand [^{18}F] altanserin, a significant decrease in 5-HT_{2A} receptor density was reported in hippocampus of both young and old depressed patients (Sheline, et al., 2004; Mintun, et al., 2004).

In recent years, a number of open-label and placebo-controlled studies have suggested that atypical antipsychotic drugs augment the clinical response to selective serotonin reuptake inhibitors (SSRI) in treatment-resistant patients (Ostroff and Nelson, 1999; Marangell, et al., 2002; Marek, et al., 2003). One common feature of these agents is their ability to occupy 5-HT_2 receptors in the brain at clinical doses and to block 5-HT_2 -mediated responses, in particular those mediated by 5-HT_{2A} receptors (Marek, et al., 2003). Studies in 5-HT_{2A} receptor knock-out mice showed a significant reduction in forced-swim test immobility (test of antidepressant drug action) that was of equal magnitude to that produced by the antidepressant mianserin and to the 5-HT_{2A} receptor antagonist, MDL100907. The lack of change in other behavioral tests including open-field locomotor activity, entries in the elevated-plus maze test, and susceptibility to pentylenetetrazole (PTZ)-

induced seizures suggested that non-specific changes in locomotor activity or neuronal excitability were not responsible for the reductions in immobility observed in the forced swim test (Sibille, et al., 1997). Altogether, these observations support a role for 5-HT_{2A} receptors in antidepressant drug action.

Schizophrenia and 5-HT_{2A} receptors:

Schizophrenia is a group of illnesses that usually begins during adolescence or young adulthood. It is characterized by two or more of the following, and each present for a significant portion of time during a 1-month period: delusion, hallucinations, disorganized speech, grossly disorganized or catatonic behavior, and negative symptoms (affective flattening, alogia or avolution). The realization in the early 1950s, that LSD can cause psychosis suggests that abnormal neurotransmission at 5-HT_2 receptors may be involved in pathophysiology of schizophrenia (Woolley and Shaw, 1954). Direct evidence supporting this hypothesis came primarily from post-mortem ligand binding studies in which a decrease in 5-HT_{2A} receptor density in the prefrontal cortex of untreated schizophrenic patients was observed (bi-Dargham, et al., 1993). Additional studies like positron emission tomography (PET) suggested a decrease in 5-HT_{2A} receptor density in schizophrenic patients taking atypical antipsychotics (Trichard, et al., 1998). However, a great deal of research on 5-HT_{2A} receptors and schizophrenia now suggests

that the changes in this receptor are complex and may be involved in both the pathology of the disorder and the effects of some antipsychotic drugs(Dean, 2003).

In general, the serotonergic system has an inhibitory influence on dopaminergic neurons and this effect appears to be mediated by 5-HT_{2A} receptors at the level of midbrain (e.g. substantia niagra and ventral tegmental area) as well as at the terminal regions of the nigrostriatal and mesocortical dopaminergic pathways (Kapur and Remington, 1996). According to Weinberger, the primary defect in schizophrenia may be the diminished dopaminergic innervention of the prefrontal cortex, which results in mesolimbic dopaminergic hyperactivity (Weinberger, 1987) . Thus, an increase in subcortical dopaminergic function may account for the positive symptoms and a decrease in cortical dopaminergic function may underlie the negative symptoms of schizophrenia. In addition, the atypical antipsychotic clozapine was reported to induce a sustained enhancement in dopaminergic tone in the medial frontal cortex (Youngren, et al., 1994), which may be attributed to antagonism of 5-HT_{2A} receptors(Meltzer, et al., 1989). The ability of 5-HT_{2A} receptor antagonists to modify behavioral states associated with excess dopaminergic activity, while having little or no effect on basal dopamine function, may be relevant to the lack of extra-pyramidal effects observed following administration of atypical antipshychotics such as clozapine (Meltzer, et al., 1989).

Furthermore, it is suggested that the atypical antipsychotic-induced internalization (sequestration of the receptor away from the cell surface) of 5-HT_{2A} receptors may be involved in the therapeutic effect of these drugs. Interestingly, antipsychotics devoid of 5-HT_{2A} receptor antagonist activity had no effect on the regulation or subcellular distribution of 5-HT_{2A} receptors in cells and in vivo. Subsequently, it is suggested that 5-HT_{2A} receptor desensitization and down-regulation plays a key role in the maintenance of the therapeutic action of antipsychotics. These studies suggest that it is important to understand the molecular changes that occur with 5-HT_{2A} receptor antagonist treatment and other mechanisms that result in desensitization of 5-HT_{2A} receptor signaling. By discovering the molecular mechanisms underlying signaling and desensitization of 5-HT_{2A} receptor, new targets for therapeutic intervention may be identified.

Signaling Pathways of 5-HT_{2A} receptor

Classically, 5-HT_{2A} receptors are coupled to $G_q/_{11}$ G alpha-proteins and mediate physiological responses by activating PLCβ, leading to the generation of IP3 and diacylglycerol (DAG)(Taylor, 1990;Conn and Sanders-Bush, 1984). IP3 mobilizes calcium from intracellular stores which then activates calcium-dependent kinases and various signaling pathways (McKune and Watts, 2001;Takuwa, et al., 1989). However, recent studies have revealed a rich diversity of coupling mechanisms of 5-HT_{2A} receptors

suggesting that these receptors can regulate a broad array of potential effectors depending on cell type, receptor population, density and types of G-proteins expressed, and the specific agonist or antagonist by which the receptor is activated.

Activation of Phospholipase A2:

The 5-HT_{2A} receptor can mediate stimulation of phospholipase A2 (PLA2); thereby generating the second messenger arachidonic acid (AA)(Tournois, et al., 1998). 5-HT_{2A} receptors can couple to PLA2 activation through two parallel signaling cascades: (1) activation of the pertussis toxin-sensitive G protein, namely $G_{\alpha i/o}$, causing the release of $G_{\beta\gamma}$, which is then free to initiate activation of the Ras-Raf-MEK-ERK signaling cascade, ultimately leading to ERK-mediated phosphorylation of cytosolic PLA2 (cPLA2); and (2) activation of receptor-coupled pertussis toxin-insensitive Gα12/13, which functions to activate Rho, and ultimately results in p-38 mediated phosphorylation of PLA2 (Chambers, et al., 2003; Kurrasch-Orbaugh, et al., 2003). In addition, because inhibition of either pathway caused a nearly identical reduction in AA release, it appears that the two pathways share a common final enzyme known as mitogen activated protein kinase (MAPK) prior to PLA2 activation. It was shown that only the 5-HT_{2A} receptor antagonist ketanserin and the PLA2 inhibitor mepacrine were able to inhibit all 5-HT-induced AA release in NIH3T3 cells (Kurrasch-Orbaugh, et al.,

2003b). Qu et al. (2003) reported that the 5-HT syndrome, characterized by head and body shakes, ear scratching, skin jerks, and forepaw tapping, involves widespread brain activation of PLA2 via 5-HT_{2A} receptors, leading to the release of the second messenger, arachidonic acid (Qu, et al., 2003). These reports suggest that 5-HT_{2A} receptors could differentially regulate the PLA2 and PLC signaling pathways depending upon the cellular environment (Nagatomo, et al., 2004).

Activation of Extracellular Signal-Regulated Mitogen-Activated Protein Kinase (ERK-MAPK):

Previously, signals generated by receptor tyrosine kinases (RTKs) and GPCRs were thought to be completely compartmentalized, with little or no cross-talk between the signaling pathways. Recently, the lines of distinction between signaling pathways used by GPCRs and RTKs have become less definite. There is a new awareness that RTKs such as the epidermal growth factor (EGF) receptor (EGFR) and GPCRs possess significant potential for cross-talk during signal initiation and propagation. The transactivation of EGFR-dependent signalling pathways upon stimulation of GPCRs, which are critical for the mitogenic activity of ligands such as lysophosphatidic acid, endothelin, thrombin, bombesin and carbachol, provides evidence for such an interconnected communication network (Prenzel, et al., 1999). 5-HT_{2A} receptor-mediated activation of ERK-MAP kinases in cells with contractile

phenotypes and vascular smooth muscle cells require PLC, L-type Ca2+ channels, and MEK1 (Florian and Watts, 1998;Watts, et al., 1998). In renal mesangial cells, activation of ERK-MAPK is mediated by PKC and activation of an NAD(P)H oxidase-like enzyme (Grewal, et al., 1999;Greene, et al., 2000). Xu et al.(2002) reported that in sheep aortic valve interstitial cells (SAVIC), 5-HT-induced activation of Erk1/2 via the MAP-kinase pathway is mediated only in part by 5-HT_{2A} receptor activity (Xu, et al., 2002). Both PKC and Src/Src-like tyrosine kinase are involved in mediating the stimulatory effects of 5-HT on Erk1/2 activity.

Besides these non-neuronal cell lines, in PC12 cells (endogenously expressing 5-HT_{2A} receptors) 5-HT-induced activation of ERK has been reported to be mediated by calcium-calmodulin and tyrosine kinases independent of PKC (Quinn, et al., 2002). 5-HT_{2A} receptors are also reported to activate Akt in a calcium dependent manner in PC12 cells (Johnson-Farley, et al., 2005). Furthermore, 5-HT_{2A} receptors are suggested to regulate activity of growth factor receptors. For example, in cultured renal mesangial cells, stimulation of 5-HT_{2A} receptors mediated activation of tumor necrosis factor-activating enzyme (TACE), the shedding of heparin-bound epidermal growth factor (HB-EGF), and consequent activation of EGF receptor. Activation of EGF receptors results in ERK activation and cell proliferation (Gooz, et al., 2006)

Taken together these reports suggest that 5-HT_{2A} receptors mediate a wide variety of peripheral and central physiological effects by activation of other signaling pathways. Further understanding of these diverse interactions of 5-HT_{2A} receptors with other signaling pathways could shed light on changes in gene expression observed with various psychotic drugs acting on 5-HT_{2A} receptors. Given the importance of 5-HT_{2A} receptors in both pathological situations and regular physiological processes, the next section will focus on various mechanisms proposed to mediate regulation of this receptor.

Regulation of 5-HT_{2A} receptors

Many GPCRs can be desensitized and down-regulated by overexposure to agonists. The 5-HT_2 receptor system is unique in that chronic exposure to either agonists or antagonists leads to receptor desensitization and down-regulation (Toth and Shenk, 1994;Toth and Shenk, 1994; Blackshear, et al., 1986; McKenna, et al., 1989). Desensitization can occur as a result of receptor uncoupling from G protein, internalization (sequestration of the receptor away from the cell surface), or down-regulation (reduced ligand-bound receptor); each has been reported for 5-HT_{2A} receptors.

Generally, G protein-linked receptor system functioning is highly regulated. These receptors are regulated by transcriptional mechanisms,

post-transcriptional mechanism such as mRNA editing and post-translational modifications of the receptors, including phosphorylation (Dohlman and Thorner, 1997; Chuang, et al., 1996; Freedman and Lefkowitz, 1996; Burns, et al., 1997). Other mechanisms also influence the functioning of G protein-linked receptor system by altering coupling of G proteins to receptors such as binding of inhibitory proteins to receptors (e.g. arrestins for adrenoceptors) (Siderowski, et al., 1996).

This section will briefly describe the following classical mechanistic models of 5-HT_{2A} receptor desensitization: I) receptor-effector uncoupling as the consequence of receptor phosphorylation and arrestin binding, II) receptor internalization that removes agonist-activated

Cell surface receptors from the plasma membrane to a membrane-associated intracellular compartment and, III) receptor down-regulation as the result of lysosomal protein degradation or reduced mRNA or protein synthesis.

I) Functional uncoupling of 5-HT_{2A} receptors:

An essential step in the desensitization of GPCRs is phosphorylation, a process that can be achieved within seconds to minutes of agonist stimulation and leads to uncoupling GPCRs from their G-proteins (Tobin, 1997). Two patterns of phosphorylation-induced desensitization have been characterized for GPCRs: homologous and heterologous desensitization. Homologous desensitization is an agonist-specific process based on the phosphorylation of

only those receptors that were activated by a given agonist. In contrast, heterologous desensitization reduces the sensitivity of receptors other than those stimulated by a particular agonist.

G-protein-coupled receptor kinase (GRK)-mediated desensitization:

As with other GPCRs, it is generally thought that the desensitization of 5-HT_2 receptors can be induced by GRKs and arrestins. In particular for the 5-HT_{2A} receptor, arrestins can bind to the third intracellular loop of the 5-HT_{2A} receptor and are co-localized with 5-HT_{2A} receptors in cortical pyramidal neurons. Furthermore, endogenously expressed 5-HT_{2A} receptors in C6 glioma cells show arrestin-dependent desensitization (Gray, et al., 2001). However, the desensitization of 5-HT_{2A} receptors in the heterologous HEK-293 cell system seems to be arrestin-independent, possibly indicating that the underlying mechanisms of homologous 5-HT_{2A} receptor desensitization are cell-type dependent (Bhatnagar, et al., 2001; Gray, et al., 2001).

Desensitization mediated by other kinases:

Desensitization can be mediated by second messenger-dependent phosphorylating proteins, e.g. protein kinase A (PKA) or C (PKC) or calcium/calmodulin-dependent kinase (CaMK). Based on the effects of PKC inhibitors (e.g., staurosporine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7)) and activators (e.g., mezerein, phorbol 12-myristate 13-acetate (PMA)), a PKC-mediated feedback system appears to mediate the

desensitization of 5-HT_{2A} receptors (Anji, et al., 2000; Kagaya, et al., 1990; Marek and Aghajanian, 1995; Rahimian and Hrdina, 1995; Rahman and Neuman, 1993). Rahimian and Hrdina (1995) further confirmed that the 5-HT_{2A} receptor can be desensitized as a result of an agonist-induced increase in PKC activity. In C6 glioma cells, which express only the PKC α and γ isoforms, treatment with 5-HT increases the levels of PKC α and γ, suggesting that both isoforms may be involved in 5-HT_{2A} receptor regulation (Anji, et al., 2000). Analogously to PKC, a second second-messenger-dependent kinase, CaMK, seems to be involved in the pathway that desensitizes 5-HT_{2A} receptors in C6 glioma cells (Akiyoshi, et al., 1993; Kagaya, et al., 1993; Rahman and Neuman, 1993).

II) Internalization of 5-HT_{2A} receptors:

It is generally accepted that 5-HT_{2A} receptors undergo an agonist-induced internalization. Rapid 5-HT_{2A} receptor internalization to endosomes after exposure to an agonist occurs via clathrin-coated vesicles in cells (Bhatnagar, et al., 2001; Berry, et al., 1996). Analogously to classical GPCR regulation, agonist-induced internalization of endogenously expressed 5-HT_{2A} receptors mediates the receptor resensitisation (Gray, et al., 2001). However, the 5-HT_{2A} receptor is differently regulated in HEK-293 cells in that 5-HT_{2A} receptor internalization is not necessary for receptor resensitisation. Gray et al (2001) showed that inhibition of 5-HT_{2A} receptor internalization in HEK-293 cells results in an increased resensitisation, suggesting novel cell-type-

specific modes of regulation for this receptor. After endosomal sorting, the 5-HT_{2A} receptors can be degraded or exported back to the plasma membrane.

III) Down-regulation of the 5-HT_{2A} receptors:

In vivo studies have shown that 5-HT_{2A} receptors are down-regulated after prolonged agonist stimulation (Eison, et al., 1989; Chaouloff, et al., 1993; Pauwels, et al., 1990). However, reports from cell culture studies are not consistent, similar results have been found in some, but not all, cell culture studies. Agonist exposure induces a down-regulation, up-regulation or no change in 5-HT_{2A} receptor density expressed in cells (Ferry, et al., 1993; Rinaldi-Carmona, et al., 1994). Grotewiel and Sanders–Bush (1994) have suggested that the cellular background in which the 5-HT_{2A} receptor is expressed appears to determine the regulation of the 5-HT_{2A} receptor density (Grotewiel and Sanders-Bush, 1994). They have further shown that after agonist pre-treatment, 5-HT_{2A} receptors are down-regulated, unaltered or up-regulated depending on which heterologous expression system was used. Although some cell culture studies showed that 5-HT_{2A} receptors are desensitized and down-regulated after exposure to agonists (Ivins and Molinoff, 1990; Van, et al., 1993), desensitization of 5-HT_{2A} receptors, in particular in heterologous expression systems, can occur without reduction in receptor density (Van, et al., 2003;Roth, et al., 1995). These results imply that 5-HT_{2A} receptor desensitization can occur with or without receptor down-

regulation, indicating that the mechanisms of 5-HT_{2A} receptor desensitization may be distinct from those involved in down-regulation and internalization.

Consistent with most data from cell culture and in vivo studies on agonist-induced regulation, agonist-induced desensitization of 5-HT_{2A} receptors can be explained as a multistep phenomenon. First, the 5-HT_{2A} receptors are uncoupled from the G-protein upon phosphorylation by one or more kinases (e.g. GRKs, PKC or CaMK) (The GRK-induced desensitization is completed by the binding of arrestins). Second, 5-HT_{2A} receptors are internalized via clathrin-coated pits, followed by transport to endosomes. After endosomal sorting, 5-HT_{2A} receptors can be shuttled back to the cell membrane or degraded. The occurrence of 5-HT_{2A} receptor down-regulation in cells seems to depend on the cell system. Although models for agonist-induced regulation of 5-HT_{2A} receptors have been formulated, the underlying mechanisms remain poorly understood. The cellular background in which the 5-HT_{2A} receptor is expressed in cells appears to determine the regulation properties of this receptor.

Paradoxical down-regulation of 5-HT_{2A} receptors by Antagonists

In vivo regulation studies showed that the adaptive changes of 5-HT_{2A} receptors after administration of 5-HT_{2A} antagonists are different from those in other receptor systems. Many reports demonstrated that chronic administration of compounds with 5-HT_{2A} antagonistic action leads to reduced

5-HT_{2A} receptor levels (i.e., the B_{max}-value was reduced and the K_D-value was unaltered after treatment with the antagonists). Studies using more selective 5-HT_{2A} receptor antagonists further confirmed the atypical down-regulation of 5-HT_{2A} receptor binding sites (Burnet, et al., 1996; Matsubara and Meltzer, 1989; Mikuni and Meltzer, 1984).

The consistent results obtained in vivo for antagonist-induced regulation of 5-HT_{2A} receptors stand in contrast to the variable results from cell culture studies. In cells, the antagonist-induced regulation of the 5-HT_{2A} receptor may vary depending on the antagonist used, host cell line and species of the receptor. Indeed, the total number of (antagonist) 5-HT_{2A} receptor binding sites was found to be decreased, increased, or unaltered by antagonist treatment (Toth, 1996; Barker and Sanders-Bush, 1993). Grotewiel and Sanders–Bush stated that in cells the effects of chronic antagonist exposure to 5-HT_{2A} receptors depend on the cell model system (Grotewiel and Sanders-Bush, 1994). In order to get a better understanding of what happens to the receptor population following prolonged drug treatment, it is important to investigate both high affinity agonist binding sites (G-protein-coupled receptors) and the total population of antagonist-labeled receptors. Apparently inconsistent findings may be related to the different radioligands used in the different studies and the accuracy with which receptor numbers are quantified. Willins et al (1999) showed that antipsychotics with high affinity for 5-HT_{2A} receptors induce a 5-HT_{2A} receptor internalization, which could

explain the paradoxical down-regulation of 5-HT_{2A} receptors (Willins, et al., 1999). In contrast, antipsychotics devoid of 5-HT_{2A} receptor antagonist activity had no effect on the regulation or subcellular distribution of 5-HT_{2A} receptors in cells and in vivo. Consequently, it is suggested that 5-HT_{2A} receptor antagonism by antipsychotics and the down-regulation of 5-HT_{2A} receptors play a role in the maintenance of the therapeutic action of the drugs.

As evident from these reports, there is no consensus model for antagonist-induced desensitization of 5-HT_{2A} receptor signaling. Hence, it is reasonable to presume that novel cellular and biochemical mechanisms are responsible for antagonist-induced internalization of 5-HT_{2A} receptors. Because of the pivotal role of 5-HT_{2A} receptor in many processes and because of its importance in atypical antipsychotic medications, further insight into mechanism of 5-HT_{2A} receptor regulation will likely have important ramifications for many areas of neurobiology and psychiatry. Therefore, it is important to understand the molecular changes that occur with 5-HT_{2A} receptor antagonist treatment and other methods that result in desensitization of 5-HT_{2A} receptor signaling.

In addition to these classical mechanisms of regulation of 5-HT_{2A} receptors, there are additional mechanisms which affect the downstream signaling pathways of 5-HT_2 receptors including those that affect the ability of the G proteins to bind to nucleotides and activate the second messenger system. One mechanism is used by a group of molecules to inhibit nucleotide

exchange on the Gα subunit of G proteins (Dohlman and Thorner, 1997). The other mechanism is to accelerate the inactivation of the GTP-Gα subunit by increasing the rate of hydrolysis of the bound GTP. Proteins that work via this second mechanism are termed GTPase-activating proteins (GAPs) such as regulators of G protein signaling (RGS) proteins (Dohlman and Thorner, 1997;Hepler, et al., 1997). Increased expression of RGS proteins leads to desensitization in several G protein-linked receptor systems (Dietzel and Kurjan, 1987; Druey, et al., 1996; Koelle and Horvitz, 1996). The next section will focus on structural and physiological aspects of RGS proteins in the context of their interaction with various GPCRs.

RGS Proteins

Coordinated regulation of heterotrimeric guanine nucleotide–binding protein (G protein) activity is critical for the integration of information from multiple intracellular signaling networks. The process of G protein inactivation is a key step that determines the duration of the signaling and therefore the extent of the cellular response. It is controlled by a specialized protein family - RGS that act to speed up the inactivation (i.e. desensitization) of the signaling pathway by accelerating the GTP hydrolysis of the G proteins. RGS proteins form a large and diverse family, initially identified as GTPase activating proteins (GAPs) of heterotrimeric G-protein G_{α}. Activation of GPCR stimulates the exchange of GDP for GTP on G_{α} to initiate heterotrimer dissociation and

activation of effector proteins that, in turn, initiate a cascade of cellular signaling events. RGS proteins participate in this process by binding directly to the activated G_{α}-GTP to serve as GTPase-activating proteins (GAPs), which limit the lifetime of G_{α}-GTP and terminate signaling events. In addition to terminating G_{α}-dependent signaling, GAP activity results in generation of G_{α}-GDP from Gα-GTP and reformation of heterotrimer and the termination of $G_{\beta\gamma}$ -mediated signaling subunits (Berman and Gilman, 1998;Shuey, et al., 1998; Willars, 2006).

Although the majority of RGS are GAPs, some RGS can affect signaling in a non-GAP manner. For example, RGS4 and RGS19 (GAIP) besides acting as GAP for $G_{\alpha q}$, can also inhibit Gαq activity through effector antagonism (competing with effector molecules for GTP bound Gα-subunit) when activated by non-hydrolysable GTP analogue, GTPγS, (Hepler, et al., 1997) and through inhibition of ALF^{4-} -mediated G-protein activation of phospholipase C (Yan, et al., 1997). Furthermore, certain RGS proteins (RGS12 and RGS14) contain a GoLoco domain that selectively bind inactive Gα-subunits, impeding GDP release and thereby limiting G-protein activation known as guanine nucleotide dissociation inhibition (GDIs) (Kimple, et al., 2001).

Classification of RGS Proteins:

RGS proteins are a family of cellular proteins with a conserved RGS domain (also called RGS box) of about 120 amino acid residues in length. It is now well recognized that the RGS play essential regulatory roles in the G protein-mediated signal transduction. There are over 20 members in the mammalian RGS family (Ross and Wilkie, 2000). Based on the similarity in sequence and features of structural domains, they are classified into various subfamilies (R4, R7, R12, RA, and RZ) (Table 1). Their structural features are listed in Table 2. While RGS proteins by definition contain at least one RGS or RGS-like domain, there is considerable diversity outside this region. The prototype of the simplest RGS protein, RGS21 is a member of the R4 subfamily and contains only a RGS domain (Von, et al., 2004). Other RGS proteins have N-and C-terminal extensions of different lengths, many of which contain domains that have either enzymatic activity or are involved in the protein-protein interactions. These domains not only influence the ability of RGS proteins to act as GAPs or effector antagonists, but also influence other aspects of the signaling mediated by GPCR activation.

Table 1- RGS proteins: Their Gα targets and tissue distribution (most recent references are listed)

Subfamily	RGS proteins	G_α Protein	Distribution	References
RZ	RGS17(RGSZ2)	$G_{\alpha z}$	ND	(de, et al., 1999)
	RGS19(GAIP)	$G_{\alpha t}$,$G_{\alpha t}$, $G_{\alpha z}$>$G_{\alpha q}$	Ubiquitous, low in brain	(Wang, et al., 1998)
	RGS20(RGAZ1)	$G_{\alpha z}$>$G_{\alpha i}$	Brain	
R4	RGS1	$G_{\alpha i}$	Lung, β- lym -phocyte	(Druey, et al., 1996a)
	RGS2	$G_{\alpha q}$	Ubiquitous	(Siderovski, et al., 1994)
	RGS4	$G_{\alpha i}$> $G_{\alpha q}$	Brain, Heart	(Seki, et al., 1998)
	RGS5	$G_{\alpha i/o}$, $G_{\alpha q}$	Ubiquitous	(Gold, et al., 1997c)
	RGS8	$G_{\alpha i}$, $G_{\alpha o}$	Brain	(Saitoh, et al., 1997)
	RGS13	ND	Lung	Gene bank
	RGS16	$G_{\alpha i}$	Retina, Liver, Pituitary	(Chen, et al., 1997)
	RGS18	$G_{\alpha i/o}$, $G_{\alpha q}$	ND	
	RGS21	ND		

R7	RGS6 RGS7 RGS9 RGS11	$G_{\alpha o}$ $G_{\alpha i}$, $G_{\alpha q}$ $G_{\alpha t}$ $G_{\alpha o}$	ND Brain Retina, Neurons Brain	(Liang, et al., 2000) (Gold, et al., 1997b) (He, et al., 1998)
R12	RGS10 RGS12 RGS14	$G_{\alpha i}$ $G_{\alpha i}$($G_{\alpha 12/13}$) $G_{\alpha i/o}$, $G_{\alpha 12/13}$	Brain Lung, Brain, Spleen ND	(Hunt, et al., 1996) (Mao, et al., 1998) (Traver, et al., 2000)
RA	Axin1 Axin2	ND ND	Thymus, Testis	(Zeng, et al., 1997a) (Berman and Gilman, 1998a)

ND: not determined, associated G_{α}-protein coupling include reports from both in cells and *in vivo* studies, some of the information presented taken from gene bank report see(De and Gist, 1999) for further details).

Table 2- Key structural features of each subfamily and reported post-translational modification:

Subfamily	Domain characteristics	Post-Translational modification
RZ	 *c, cysteine string, RGS domain	Palmitoylation
R4	 *RGS3 has long N-terminus	Palmitoylation (RGS3,4,16) Ubiquitination (RGS4,16) Phosphorylation (RGS2,3,4,16,18)
R7	 *RGS9 has long C-terminus DEP, domain found in Dishevelled; GGL, Gγ-like domain;R7H, R7 homology	Palmitoylation (RGS7) Ubiquitination (RGS7) Phosphorylation (RGS7,9)
R12	 *RGS10 has only RGS domain *RGS14 does not have PDZ &PTB domain PDZ, post synaptic density protein (PSD95), Drosophila disc large tumor	Palmitoylation (RGS10) Phosphorylation (RGS10,14)

	suppressor (DlgA), and zonula occludens-1 protein (zo-1); PTB, phosphotyrosine binding domain; CC, coiled-coiled region	
RA	 GSK3-BD, glycogen synthetase kinase 3b-binding domain; βCat-BD, β-catenin binding domain; PP2A, protein phosphatase 2A homology domain	Phosphorylation (Axin1)

*For further information please refer to (Willars, 2006).

In addition to well-recognized protein domains, regions or motifs, N- and/or C-terminal extensions may well regulate their function by determining their sub-cellular localization. N-terminus extensions have been proposed to mediate membrane localization of many RGS proteins and the C-terminus acts as affinity adapters increasing the affinity of RGS proteins for their respective G_{α}-proteins.

Regulation of RGS Proteins:

In addition to structural diversity, RGS proteins are subjected to a number of post-translational modifications including phosphorylation, palmitoylation, and ubiquitination (Table 2). However, no consistent effect of

phosphorylation on RGS protein functions is suggested. Reports of both enhanced (Tang, et al., 2003;Pedram, et al., 2000) or reduced GAP activity (Cunningham, et al., 2001;Chen, et al., 2001;Chen and Lin, 1998), altered sub-cellular localization (Burgon, et al., 2001), protein stability (Derrien, et al., 2003), and altered ability of G-protein α-subunit to enhance RhoGEF activity have been shown (Suzuki, et al., 2003). Several RGS proteins are palmitoylated on multiple sites but it is yet to be established if and how palmitoylation of specific residues is regulated in the cellular context and how this influences the function of RGS proteins (Willars, 2006). Ubiquitination, i.e. the addition of ubiquitin to proteins, is associated with degradation of proteins by 26S proteosome. Among RGS proteins, RGS7 (Kim, et al., 1999), 4, and 16 (Davydov and Varshavsky, 2000) are reported to undergo degradation by this method.

RGS proteins in Pathophysiology of Diseases:

Recently, several studies have linked RGS proteins to various disease conditions. The following discussion will highlight the role of RGS proteins primarily in psychiatric disorders. Studies investigating changes in gene expression have extensively and consistently reported a decrease in RGS4 mRNA in prefrontal cortex, motor cortex, and visual cortices of schizophrenic patient (Chowdari, et al., 2008;Levitt, et al., 2006;Mirnics, et al., 2001;Chowdari, et al., 2002). However, similar studies in patients with major

depressive disorders do not show any change in mRNA levels of RGS4, suggesting that alteration in RGS4 expression is specific to schizophrenia. These findings along with reports of atypical antipsychotic-induced decreases in activity of 5-HT_{2A} and D2 receptor signaling highlights the notion that RGS4 could be a risk factor for developing schizophrenia.

Studies specifically looking at RGS2 and RGS9 demonstrated that reducing the levels of these proteins have an effect on response to morphine. Knocking down RGS2 makes morphine less potent while decreasing RGS9 potentiates analgesic effects of morphine(Garzon, et al., 2001). These and other studies have suggested that RGS proteins play an important role in altering effects of certain drugs of addiction because of their ability to modulate signal desensitization and thereby affecting potency of a compound. It has been proposed that small molecules that inhibit RGS protein/Gα interactions could be developed as novel drugs to potentiate the actions of endogenous neurotransmitters in various disease states such as Alzheimer's.

RGS proteins as Potential Drug Target:

Many drugs act on receptors coupled to heterotrimeric G proteins (GPCRs). Historically, drug discovery has focused on agents that bind to the receptors and either stimulate or inhibit the receptor-initiated signal. This is an approach that is both direct and logical, and has proven extremely fruitful in the past. However, as our understanding of G-protein signaling has

increased, novel opportunities for drug development have emerged. GTPase-accelerating protein activity is a general feature of RGS proteins, and serves to facilitate the inactivation of the G protein rather than the receptor. Thus, agents that bind and inhibit RGS proteins could modulate endogenous neurotransmitter and hormone signaling, in a manner analogous to neurotransmitter uptake inhibitors.

In addition, what makes many RGS proteins such attractive new drug targets is their unique capacity to modulate G protein signaling combined with their highly regionalized localization, most notably within the central nervous system (Gold, et al., 1997). Since their initial discovery, RGS proteins have been extensively studied as important new drug targets (Zhong and Neubig, 2001; Neubig, 2002; Neubig and Siderovski, 2002). Alternatively, such therapeutic agents could be used to boost the effects of existing GPCR-directed drugs by decreasing the therapeutic dose needed while increasing the agonist's regional specificity, thereby reducing unwanted side effects (Neubig and Siderovski, 2002). The design of small molecules that block or mimic RGS protein/receptor interactions could become a highly specific therapeutic tool that is effective only in those cell types in which both the RGS protein and the receptor are localized.

Atypical Antipsychotics

Schizophrenia is a debilitating disorder that affects 1% of the world's population, often at an early age (Andreasen, et al., 1995; Ho, et al., 2003; Philip, et al., 2008). While schizophrenic patients have various abnormalities of perception, thought, language, or affect, many patients develop cognitive deficits and most show marked and long lasting impairments in social functioning (positive symptoms) (Prell, et al., 1996). Despite intense efforts, the etiology of schizophrenia has remained elusive. A promising route of investigation into the cause of the disease is to study how antipsychotic drugs alter brain function in schizophrenia. An improved understanding of how antipsychotic drugs convey their therapeutic effects during the treatment of schizophrenia can help to unravel neural mechanisms involved in the pathophysiology of schizophrenia. In the past, a similar approach has generated a prominent model of schizophrenia, the dopamine hypothesis (symptoms of schizophrenia (like psychoses) to a disturbed and hyperactive dopaminergic signal transduction) (Matthysse, 1973).

The number of therapeutic agents available for the treatment of schizophrenia have grown and diversified in the last half century since the advent of chlorpromazine and the beginning of the pharmacologic era in psychiatry. Over the past decade, much of the attention regarding the treatment of schizophrenia and related psychotic disorders has focused on a new class of antipsychotic medications (Weiden, et al., 2006;Mathews and

Muzina, 2007). The reintroduction of clozapine was a major step forward, and led to the proliferation of atypical or second generation antipsychotic drugs, including risperidone, olanzapine, quetiapine, ziprasidone, and sertindole (Lieberman, et al., 1989; Meltzer and Nash, 1991; Kane and Freeman, 1994). In fact, there is growing evidence that most of these drugs offer advantages over typical or first generation antipsychotic such as greater improvement in negative symptoms i.e. lack of behaviors (such as emotion, speech, social interaction, and action), cognitive impairment, relapse prevention, functional capacity and quality of life with fewer extra pyramidal side effects and tardive dyskinesia (Farah, 2005; Brooke, et al., 2005; Pierre, 2005; Seeman, 2002). Although, these improvements are substantial considering the improvement in overall quality of life, other distressing side effects like weight gain, hyperglycemia, and dyslipidemia are widely reported with atypical antipsychotic drugs (Roerig, et al., 2008; Henderson, 2007; Ferraioli, et al., 2004). Furthermore, the full clinical profile is still being studied in terms of the extent of therapeutic efficacy and adverse effects, on a variety of other outcomes including cognition, affect, subjective response, and social and vocational functions.

All atypical antipsychotic drugs share a similar receptor binding profile, high affinity for dopamine receptors D_1, D_2, D_4, 5-HT receptors 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, α1-adrenergic, histamine H1, and five muscarinic receptor subtypes and low affinity for α2-adrenergic receptors and relatively low affinity

for $5HT_1$ subtypes, gamma aminobutyric acid A ($GABA_A$), β-adrenergic receptors, and benzodiazepine binding sites (Kendrick, 1999). This binding profile is consistent across rat and human brain, and in cell lines. This receptor binding profile of atypical antipsychotic drugs is also correlated with the antidopaminergic, antiserotonergic, and antimuscarinic activity observed in animal models and predicts atypical antipsychotic activity in human (Bymaster, et al., 1996a;Bymaster, et al., 1996b). However, antipsychotic properties of atypical antipsychotics were more commonly attributed to the potent antagonism/inverse agonism of 5-HT_{2A} than dopamine D2 receptors (Arora and Meltzer, 1994; Masellis, et al., 1995). Since 5-HT_{2A} receptors are focus of this dissertation, the following section will discuss findings suggesting that 5-HT_{2A} receptors are pivotal in the action of atypical antipsychotic drugs. Over the years, intensive research on the new antipsychotic drugs has led to a greater understanding of the biochemical effects of these drugs, however, the pharmacological mechanisms underlying their therapeutic properties remain to be identified. Understanding the mode of action of the atypical antipsychotic drugs will be useful in exploring the pathophysiology of schizophrenia and other psychotic disorders and for the development of potential new therapeutic targets.

Table 3: Different classes of antipsychotic drugs and target receptors:

Drugs	Class	Receptor affinity	Side effects
Haloperidol	Typical	D_1 & **D_2**, 5-HT_2 and α_2	EPS, Prolactin Weight gain
Clozapine	Atypical	D_1, D_2, 5-HT_2, α_2, **H1, M1**	Weight gain, Postural hypotension, Sedation, Anti-cholinergic effect, Granulocytosis
Olanzapine	Atypical	D_1, D_2, **5-HT_2, H1, M1**	Weight gain, Sedation, Dizziness, Transient elevation of Liver transaminases
Risperidone	Atypical	D_1, D_2, **5-HT_2**, H1, α_2	Weight gain, Seizure, Dystonia, Anxiety, Prolactin
Ziprasidone	Atypical	D_1, **D_2**, **5-HT_2**, H1	Weight gain, Seizure, Dystonia
Sertindole	Atypical	D_1, **D_2**, **5-HT_2**, H1, M1	Prolonged QT interval
Quetiapine	Atypical	D_1, **D_2**, 5-HT_2, **H1, M1**	Anti-cholinergic effects

*For further information please refer to (Kendrick, 1999).

Mechanism of Action of Atypical Antipsychotic Drugs:

Woolley and Shaw (1954) proposed that because hallucinogens are structurally related to LSD, and because schizophrenia is a disease exemplified by hallucination, then schizophrenia must be a disease involving 5-HT (Woolley and Shaw, 1954). This "serotonin hypothesis of schizophrenia" was later discarded in favor of the 'dopamine hypothesis of schizophrenia' because of several observations that implicated dopamine (DA) in antipsychotic drug action (Carlsson and Lindqvist, 1963;Creese, et al., 1976). Additionally, clinical evidence suggested that the syndrome elicited by hallucinogens was quite distinct from that seen in schizophrenia (Hollister, et al., 1962). Although, drugs which are developed to target dopamine D2 receptors "typical antipsychotic drugs" are effective in treating certain core symptoms of schizophrenia, they also frequently induce serious side effects including negative symptoms, cognitive impairment, extra pyramidal side effects and tardive dyskinesia.

However, the recognition that clozapine was effective without having motor side effects or appreciable affinity for either D1 or D2 dopamine receptors led to a reevaluation of dopamine hypothesis of schizophrenia and antipsychotic drug actions. Importantly, clozapine has a higher affinity for 5-HT_{2A} receptors than for either the D1 or D2 receptors. Although, clozapine was superior in treating psychosis, it was withdrawn after wide spread reports of agranulocytosis, a potentially fatal blood dyscrasia. In fact, the superior

efficacy and lack of motor side effects eventually led to the search for other atypical antipsychotic drugs without the propensity to induce agranulocytosis. Like clozapine, all new members of this class bind with high affinity to 5-HT_{2A} receptors. These findings renewed interest in the role of 5-HT receptors in antipsychotic drug actions and the role of 5-HT in the pathophysiology of schizophrenia (Roth, et al., 1995).

Role of 5-HT_{2A} receptors in Antipsychotic Treatment:

Altar et al. (1986) was the first to systematically examine the role of 5-HT_{2A} receptors in atypical antipsychotic drug actions (Altar, et al., 1986) . They found that several putative atypical antipsychotic drugs displaced [^{3}H] spiperone binding to 5-HT_{2A} receptors more easily than to D2 dopamine receptors. Additionally electrophysiological studies have also shown that atypical antipsychotic drugs have potent 5-HT_{2A} receptor antagonist activity (Charney, et al., 1988). Further studies by Meltzer et al. (1989) demonstrated that a large number of atypical antipsychotic drugs were characterized by having higher affinities for 5-HT_{2A} receptors than for D1 or D2 receptors (Meltzer, et al., 1989). These studies have led in part to the development of a new class of atypical antipsychotic drugs with higher affinity for 5-HT_2 over D2 receptors.

Atypical antipsychotic drugs reduce positive symptoms by blocking dopamine D_2 receptor in the mesolimbic systems without affecting dopamine

receptors in nigrostriatal system. Their effect on negative symptoms may be mediated through the enhancement of dopamine activity in the pre-frontal cortex, either by selective binding to dopamine receptor subtypes or through antagonism at 5-HT_2 receptors. Consistent with the 5-HT_2:D_2 antagonism hypothesis of schizophrenia, numerous reports have consistently observed that antipsychotic effects of clozapine, olanzapine, sertindole, and quetiapine are mediated by their ability to block 5-HT_{2A} receptor without excessive blockade of D_2 receptors (Nyberg, et al., 1997; Kasper, et al., 1999; Kasper, et al., 1999). The strong antagonism of 5-HT_{2A} receptor by these drugs was further confirmed with MDL100907 (a highly specific antagonist of 5-HT_{2A} receptor) (Maixner, et al., 1998). Furthermore, His52Tyr allele of the 5-HT_{2A} receptor, which accounts for 10-20% of the schizophrenia patients is associated with the poor response to clozapine, again confirming role of 5-HT_{2A} receptor antagonism in the action of clozapine and other drugs of this class (Masellis, et al., 1998; Arranz, et al., 2001).

Effect of 5-HT_{2A} receptor Antagonism and D2 receptor Function:

It had been proposed that the usefulness of 5-HT_{2A} receptor antagonists in treating psychosis results from their influence on dopaminergic activity in mesolimbic and mesostriatal system. Increased dopaminergic activity in the nucleus accumbens and other mesolimbic and possibly cortical regions may contribute to the positive symptoms, whereas increased

dopaminergic activity in the striatum would diminish extrapyramidal side effects (EPS). There is considerable evidence suggesting that 5-HT_{2A} receptors modulate activated but not basal mesolimbic DA activity (Gleason and Shannon, 1997; Martin, et al., 1998). Therefore, drugs that block the effect of excessive, but not basal, D2 receptor activity may be the most effective clinically. This hypothesis was further supported by reports that MDL100907 has been found to diminish the increase in DA efflux in the nucleus accumbens produced by either haloperidol or S-sulpiride (Liegeois, et al., 2002;Ichikawa and Meltzer, 1995). Therefore, it is very likely that 5-HT_{2A} receptor antagonism may have antipsychotic action when dopaminergic activity is slightly or moderately increased.

5-HT_{2A} receptor Antagonism and Glutamate Levels:

Besides dopamine, 5-HT_{2A} receptors on cortical pyramidal neurons are also suggested to play a crucial role in psychosis by modulating intracortical and cortical-subcortical glutaminergic neurotransmission (Jakab and Goldman-Rakic, 1998). Aghajanian and Marek have proposed a link between the glutamate hypothesis of schizophrenia and hallucinogen hypothesis based on the observation that stimulation of 5-HT_{2A} receptors on layer V pyramidal cells increases the frequency of postsynaptic potentials (PSP) and this was blocked by AMPA/Kinate glutaminergic antagonist LY293558 (Marek, et al., 2000; Aghajanian and Marek, 2000).

Effects on Gene Expression and Cell Signaling:

As discussed in previous sections, atypical antipsychotic drugs cause desensitization of 5-HT_{2A} receptor signaling both in cell culture and in rat brain. These drugs also alter expression of a host of genes including 5-HT_{2A} receptors (Burnet et al., 1996). However, whether changes in gene expression and desensitization have any cause and effect relationship is currently not known. The following section will review the literature reporting various signaling pathways activated or stimulated by these drugs. However, in line with the focus of this dissertation, the following discussion will be limited to the change in 5-HT_{2A} receptor expression.

In situ hybridization and autoradiography studies reported selective decreses of 5-HT_{2A} receptor mRNA and the density of [^{3}H] ketanserin binding in cingulate and frontal cortex of rats injected for 14 days at the dose of 25 mg/kg/day clozapine. In the same study, no change was observed in 5-HT_{1A} receptor expression and 5-HT_{2c} receptor messenger RNA suggesting that 5-HT_{2A} receptor is the primary target of clozapine and similar drugs (Burnet et al., 1996). Subsequent studies by Buckland et al. (1997) in whole brain of animals treated with clozapine (10 mg/kg/day) for 32 days also reported a decrease in 5-HT_{2A} mRNA levels in hippocampus, brainstem and midbrain. In contrast, 4 day treatment with clozapine increased 5-HT_{2A} mRNA in nucleus accumbens (Buckland, et al., 1997). The increase in mRNA levels after acute treatment may be a short-term adaptive response to the antagonistic effect of

these drugs. Additional studies with olanzapine using both acute (24 and 48 h) and subchronic (16 day) treatment in rats reported a decrease in 5-HT_{2A} receptor density both in frontal cortex and blood platelets without any changes in receptor affinity (Padin, et al., 2006).

Activation of Signaling Pathways:

Numerous reports have also suggested activation of different signaling pathways with atypical antipsychotic drugs in cell culture. Clozapine was reported to activate glycogen synthase kinase-3β(GSK-3β) in SH-SY5Y cells (human neuroblastoma) by specific Wnt signal pathway independent of AKT(Kang, et al., 2004). Other reports have suggested AKT-dependent activation of GSK-3β by inhibiting calcium calmodulin kinase (CaM-Kinase) with clozapine (Shin, et al., 2006). Therefore, it seems that these effects are more likely to be cell type specific. However, no effort was made to link whether these effects are a consequence of clozapine or olanzapine-induced changes on target monoamine neurotransmitter receptors. The interaction/interplay of different signaling cascades has been well studied in various disease processes such as cancer. However, such interactions are relatively unknown in various neuropsychiatric and other CNS disorders.

In addition, $5HT_{2A}$ receptors are reported to mediate activation of the JAK-STAT pathway in multiple cell culture systems (Guillet-Deniau, et al., 1997; Banes, et al., 2005). Activation of the JAK-STAT pathway transmits

information received from extracellular polypeptide signals, through transmembrane receptors, directly to target gene promoters in the nucleus, providing a mechanism for transcriptional regulation. Evolutionarily conserved in eukaryotic organisms from slime molds to humans, JAK-STAT signaling appears to be an early adaptation to facilitate intercellular communication (Rawlings, et al., 2004). Thus, the JAK-STAT pathway seems to be an interesting prospect which might impart to atypical antipsychotics the ability to induce change in gene expression; however, this hypothesis needs to be tested. The following section will describe JAK-STAT pathway in more detail.

JAK-STAT Pathway

Characterization of the ability of interferon-α (IFN-α) to rapidly induce genes led to the discovery of the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway (Schindler and Darnell, Jr., 1995;Ihle, 1995). The JAK-STAT pathway is one of a handful of pleiotropic cascades that transduce a multitude of signals for development and homeostasis in animals, from humans to flies (Heim, 1999;Pires-daSilva and Sommer, 2003). In mammals, the JAK-STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors (Duncan, et al., 1997). JAK activation is reported to stimulate cell proliferation, differentiation, cell migration, and apoptosis (Igaz, et al., 2001; O'Shea, et al., 2002). The JAK-STAT signaling was discovered almost 15 years ago with the

identification of a novel class of interferon-activated transcription factors (Harpur, et al., 1992; Shuai, et al., 1992).

Although, first described as the signal-transducing pathway of interferons, the JAK-STAT system was soon discovered to be utilized by numerous cytokines and non-immune signaling pathways (growth factor, hormones etc). Besides cytokines, various receptors are also reported to activate the JAK-STAT pathways (Table 4). Since its discovery, extensive studies have characterized the core components of the JAK-STAT signal transduction pathway. It includes a wide and diverse range of extracellular ligands and transmembrane receptors, four genes for JAKs and seven genes coding for STATs (Kisseleva, et al., 2002).

Activation of the JAK-STAT pathway requires binding of an extracellular ligand to a transmembrane receptor, results in the activation of receptor-associated JAKs. Activated JAKs then autophosphorylate and phosphorylate their associated receptors to generate docking sites for the SH2 domains of STATs. According to the established model, STATs are normally present in the cytoplasm as inactive monomers before being recruited to receptor/JAK complex. However, it has also been shown that STATs constitutively shuttle between the cytoplasm and nucleus before being retained in the nucleus following activation (Meyer and Vinkemeier, 2004). Once bound to the receptor/JAK complex, STAT proteins are cross-phosphorylated and form either a hetero- or homo-dimer. Stabilized by the

interaction between the SH2 domain of one molecule and the phospho-Tyr of the other molecule, the STAT-STAT dimer translocates to nucleus where it binds to a palindromic DNA sequence at the promoter of target genes to modulate transcription (as shown below).

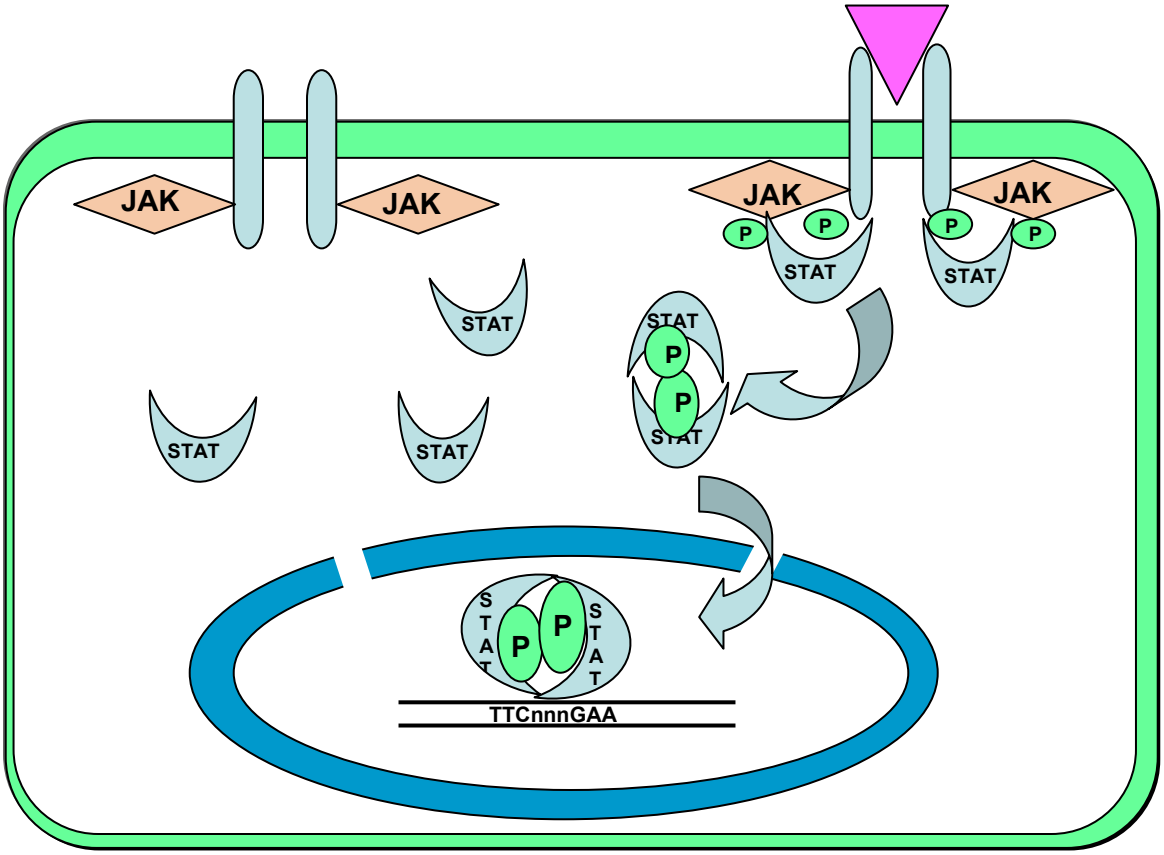


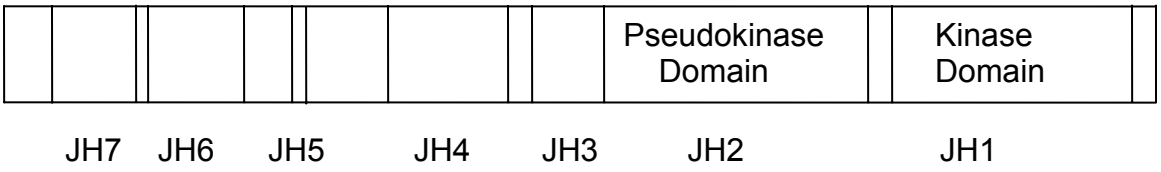
Figure 4: The Model of JAK-STAT Signaling: Pre-dimerised complexes of a receptor and JAKs are activated following ligand binding. Phosphorylation of the JAKs and the receptors generate docking sites for the normally cytosolic STATs that are recruited to the active complex. Following phosphorylation of the STATs, a STAT-STAT dimer forms then translocates to the nucleus and binds to a palindromic DNA sequence at the promoter of a

target gene. Although STAT-STAT dimer formation can occur prior to pathway stimulation, only complexes activated by Tyr-phosphorylation appear to induce target gene expression (Braunstein, et al., 2003).

Diversity of JAK-STAT Signaling:

So far four members of JAK (JAK1-4) and seven members of STAT (STAT1-7) have been identified. The JAKs range from 120-135 kDa, each of them contains a characteristic feature of two tandem kinase domains, referred to as JAK homology (JH) domains 1 and 2. Although, the most carboxy terminal domain, JH1, appears to be catalytically active, both JH1 and JH2 are required for full activation. JAKs share five additional homology domains, JH3-7. The most amino terminal domains JH6 and JH7 are believed to be important in receptor association(Schindler, 1999).

Structure of JAKs:



Structure of STATs:

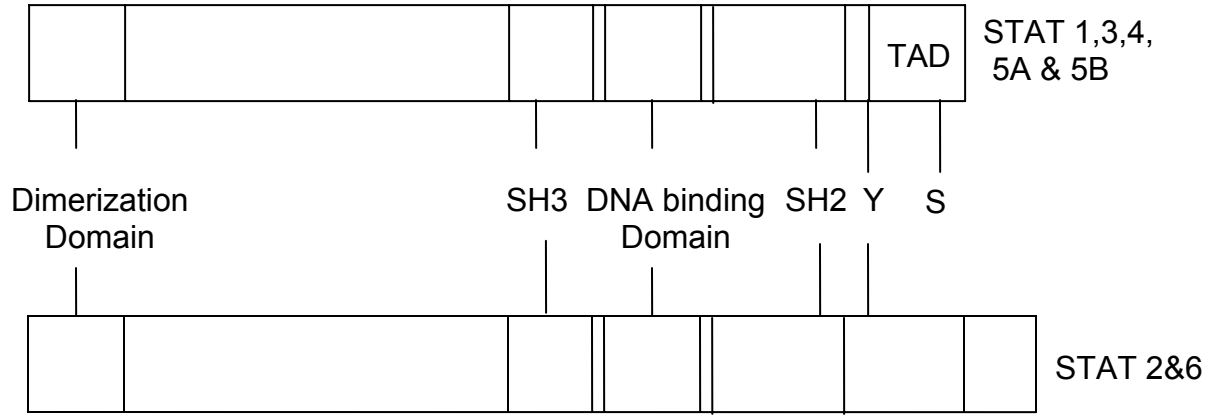


Figure 5: Structures of JAKs and STATs: The JAKs share seven regions of high homology, JH1-JH7. JH1 has been shown to encode the kinase. JH2 represents a pseudokinase domain, which appears to regulate JH1 catalytic activity. JH3 - JH7 have been implicated in receptor association. Likewise, the STATs share several conserved domains, including an amino-terminal domain (NH_2), a coiled-coil domain, the DNA-binding domain, a linker domain, an SH2 domain, and a tyrosine activation domain (TAD). The carboxy-terminal transcriptional activation domain is conserved in function, but not in sequence.

Out of seven STAT members 2 & 6 are 850 amino acids long whereas others are shorter, around 700 amino acids. Phosphorylation at tyrosine (705Y) residue facilitates dimerization with other STATs through the SH2 domain. C-terminus contains a transcriptional activation domain (TAD) and also a serine residue (S) which upon phosphorylation modulates transcriptional activity. DNA binding domain is localized between SH2 and SH3 sites.

Interaction of JAK-STAT with other GPCRs:

Recently, the interaction of various members of G-protein coupled receptor (GPCR) family with different JAKs and STATs has been a focus of investigation. Guillet-Deniau et al. (1997) reported association of JAK2 with 5-HT_{2A} receptor in skeletal muscle cells (Guillet-Deniau, et al., 1997). 5-HT stimulation of 5-HT_{2A} receptor initiated a rapid and transient tyrosine phosphorylation of JAK2 kinase. They also reported association of 5-HT_{2A} receptor and STAT3 with JAK2 by co-immunoprecipitation suggesting JAK-STAT pathways could be attributed to some of the effects mediated by 5-HT_{2A} receptor signaling.

Additional studies by other groups also identified interaction of JAK2 and activation of STAT3 with Angiotensin II (Hunt, et al., 1999) and , JAK-STAT activation by arginine-vasopressin in vascular smooth muscle cells (Levy and Granot, 2006), and 5-HT stimulation of JAK-STAT in vascular

smooth muscle cells (Banes, et al., 2005). However, except for one report of involvement of small GTPases Rho and Rac for activation of JAK2 with thrombin and angiotensin (Pelletier, et al., 2002), not much is known about the mechanism of JAK activation by GPCR agonists. Some of the other receptors which have shown to activate various JAKs and different STATs are listed below:

Table 4: Cytokine and non cytokine receptors activate different JAKs and STATs:

Receptor	**Ligands**	**JAK**	**STAT**
GPCR	Chemokines-SDF, Thrombin AngiotensinII, Endothelin, 5-HT	JAK1,2 , &3	STAT1,2,3, &5
Single chain-non kinase RTK	EGF, PDGF, GH, PRL, CSF-1, EPO	JAK1 & 2	STAT1, 3,& 5
Heterotrimeric- non kinase RTK	IL-2,3,6,10 and interferon	JAK1,2,&3	STAT1,2,3, &5

Regulation of the JAK-STAT Signaling Pathway:

A number of regulatory layers modulate this signaling pathway including both positive and negative regulators. JAK activation is dependent on tyrosine phosphorylation. Therefore, it is not surprising that two related SH2 domain containing phosphatases (as depicted in domain structure),

SHP1 and SHP2, are reported to negatively regulate JAK activity. Other reports have also suggested direct interaction and dephosphorylation of JAKs and STAT5 by SHP1 and SHP2 (Marrero, et al., 1998;Yu, et al., 2000). Besides SHPs, recent reports have also implicated membrane associated phosphatases like CD45 in negatively regulating JAK-STAT signaling stimulated by IL-3, IL-4, EPO, and IFN-γ (Irie-Sasaki, et al., 2001).

The suppressor of cytokine signaling (SOCS) are a family of STAT target genes that directly antagonize STAT activation. The founding member of this class CIS-1 (cytokine-inducible SH2 containing protein) is reported to block STAT receptor recruitment (Yoshimura, et al., 1995). Gene targeting studies with various STATs have reported that deletion of STAT1 & 3 is embryonic lethal (Naka, et al., 1998;Marine, et al., 1999a;Marine, et al., 1999b) and STAT2 null mice developed gigantism (Igaz, et al., 2001). Protein inhibitor of activated STATs (PIAS), PIAS 1 & 3 bind with activated STAT and blocks their ability to bind DNA (Gross, et al., 2001; Liu, et al., 1998).

Biological and Clinical Significance:

Investigation into the importance of the JAK-STAT pathway in vivo is hampered by the lethality of knockouts for JAK1 and JAK2; the only viable phenotypes is JAK3 (Nosaka, et al., 1995). However, JAK3 has only limited expression compared to the widely expressed JAK1 and JAK2. Thus, the role of JAK 1 and 2 during embryonic development is still not known. JAK3

knockout mice suffer from murine SCID (severe combined immune deficiency) that severely affects both the B- and T-cell population. These mice develop a phenotype which is very similar to ones lacking common γ-chain, suggesting it affects the same signaling pathway regulating assembly of various IL receptors (IL2, 4, 7, 9 &15) (Sugamura, et al., 1996; Cao, et al., 1995). Among STAT knockout models, all of them are viable except STAT3. Separate knockouts of STAT1 and STAT2 knockouts develop viral infections suggesting they might play a role in interferon signaling (Levy and Darnell, Jr., 2002; Park, et al., 2000). STAT4 and STAT6 influence differentiation of T-cells (Levy, 1999). The STAT5a knockout model does not show any severe abnormality except defects in mammary gland development. The STAT5b knockout model confirmed its role in sexually dimorphic growth hormone action (Liu, et al., 1996;Udy, et al., 1997).

Most of diseases associated with malfunctioning of the JAK-STAT pathway were initially linked with lymphohaemopoietic neoplastic disorders but recently their role in myocardial hypertrophy and bronchial asthma are recognized. Fusion proteins of JAK2 and Tel (an Ets family transcription factor) were found in some acute lymphocytic leukaemias (Lacronique, et al., 1997). Human T-cell lymphotrophic virus 1 infected T cells (HTLV-1) sustain activation of the JAK-STAT pathway and is linked to malignant transformation (Takemoto, et al., 1997). Over activity of STAT3 was reported in multiple myeloma, mycosis fungoides, and in chronic myelogenous leukemia (Catlett-

Falcone, et al., 1999; Nielsen, et al., 1999; Chai, et al., 1997). In addition to neoplastic diseases, the human SCID phenotype is in part also associated with defects of JAK-STAT pathway (Macchi, et al., 1995; Cacalano, et al., 1999).

De-Fraja et al extensively characterized expression and regulation of JAK-STAT in various brain regions (De-Fraja, et al., 1998; Cattaneo, et al., 1998). In CNS, nitric oxide (NO) plays an important role during development and progression of diseases like multiple sclerosis (MS), Parkinson's disease, and Alzheimer's disease. The JAK-STAT pathway regulates inducible nitric oxide synthetase (iNOS) expression, which regulates NO production, and thereby plays an important role during pathogenesis of these disorders (Dell'Albani, et al., 2001;Dalton, et al., 1993;Dell'Albani, et al., 2001b). Berhow et al. (1996) reported that some of the effects of chronic cocaine on ventral tegmental area (VTA) dopaminergic neurons are mediated directly by the JAK-STAT pathway (Berhow, et al., 1996). Additionally, activation of the JAK-STAT pathway in response to IL-6 induction in spinal cord injury was also reported (Yamauchi, et al., 2006). In conclusion, the JAK-STAT pathway is now being recognized as an important regulatory mechanism in CNS development, function and disease progression.

Purpose of the Present Study

Extensive literature now suggests that changes in 5-HT_2 receptors in the CNS are associated with various cognitive and mood disorders including schizophrenia, depression, anxiety, and bipolar. Studies indicate that an increased function of the 5-HT_{2A} receptor system may be associated with disorders such as depression (D'haenen, et al., 1992; Hrdina, et al., 1993) while a reduction in 5-HT_{2A} receptors has been associated with other neurological and psychiatric disorders (Gurevich and Joyce, 1997; Joyce, et al., 1997). Additionally, it has been proposed that dysregulation of 5-HT_{2A} receptor-mediated signaling may contribute to the pathogenesis of schizophrenia and related diseases. In fact, chronic treatments with antagonists that reduce 5-HT_{2A} receptor density and/or efficacy have been used clinically to treat these psychiatric disorders, such as schizophrenia (Blier and De, 1999; Aghajanian and Marek, 2000).

Although antipsychotics bind to their target receptors soon after administration, complete therapeutic benefits of antipsychotic medication take weeks to be realized (Agid, et al., 2003; Hyman and Nestler, 1996; McDermott, et al., 1991). This often results in prolonged treatment trials for individual patients until a particular drug is found therapeutically effective. To improve treatment outcome, we need to understand the mechanisms underlying the delay in the full clinical effects of antipsychotic drugs. The clinical potency of atypical antipsychotic drugs is directly correlated with their

ability to inhibit 5-HT_{2A} receptors, but this relationship cannot explain their delayed action. With the chronic use of atypical antipsychotic drug, 5-HT_{2A} receptor signaling is desensitized. Thus, it is possible that neuroadaptive mechanisms could possibly lead to alterations in the regulation of 5-HT_{2A} receptor signaling and would be responsible for this delayed clinical response. Thus, it is important to investigate how 5-HT_{2A} receptor signaling is regulated and discover the cellular pathways underlying desensitization after treatment with atypical antipsychotics such as olanzapine or clozapine.

Atypical antipsychotic drugs are inverse agonist of 5-HT_{2A} receptors in vivo in patients, and animal pharmacological studies are consistent with the notion that the 5-HT system may serve as one of the regulators of dopaminergic tone in vivo. It therefore becomes important to understand the molecular changes that occur with 5-HT_{2A} receptor antagonist treatment and other methods that result in desensitization of 5-HT_{2A} receptor signaling. With better understanding of the molecular mechanisms underlying signaling and desensitization of 5-HT_{2A} receptor signaling, new targets for therapeutic intervention may be identified.

CHAPTER III

OLANZAPINE INCREASES RGS7 PROTEIN EXPRESSION VIA STIMULATION OF THE JANUS TYROSINE KINASE-SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (JAK-STAT) SIGNALING CASCADE

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Abstract

Atypical antipsychotics such as olanzapine have high affinity for multiple monoamine neurotransmitter receptors and are the main stay of pharmacological therapy for treatment of schizophrenia. In addition to blocking monoamine receptors, these drugs also affect intracellular signaling cascades. We now report that 24-hour treatment with 300nM olanzapine causes desensitization of serotonin $(5\text{-HT})_{2A}$ receptors in A1A1v cells, a rat cortical cell line, as indicated by a reduction in inositol phosphate accumulation following stimulation with a $5\text{-HT}_{2A/2C}$ receptor agonist [(−)-1-(2,5-dimethoxy-4-Iodophenyl)-2-aminopropane HCl] (DOI). Olanzapine treatment for 24 hours increased the levels of 5-HT_{2A} receptors in both cytosol (234±34% of control level) and membrane fractions (206±14% of control levels) and RGS7 proteins in both cytosol (193±32% of control levels) and membrane fractions (160±18% of control levels) as measured on western blots. Increased phosphorylation of a tyrosine kinase JAK2, and increased

phosphorylation and nuclear translocation of STAT3 with 24-hour olanzapine treatment demonstrates activation of the JAK-STAT signaling cascade. Pretreatment with a JAK inhibitor, AG490, prevented the olanzapine-induced increase in membrane RGS7 protein levels; AG490 alone had no effect on RGS7 protein levels. We verified that treatment with AG490 reduced phosphorylation of JAK2, and inhibited the nuclear localization of phospho-STAT3. Interestingly, treatment with the JAK inhibitor had no effect on 5-HT_{2A} receptor protein levels. These data suggest that olanzapine-induced activation of the JAK-STAT signaling cascade causes increased expression of RGS7 protein, which in turn could mediate desensitization of 5-HT_{2A} receptor signaling caused by olanzapine since RGS7 binds to $G\alpha_q$ protein and accelerates GTP hydrolysis.

Introduction

Atypical antipsychotics are widely prescribed for the treatment of schizophrenia. They are classified as atypical because of their ability to achieve antipsychotic effects with lower rates of extrapyramidal side effects compared to first generation antipsychotics such as haloperidol. In addition, selected atypical antipsychotics also improve certain aspects of cognitive function in schizophrenic patients, whereas typical antipsychotics may worsen cognition (Meltzer, et al., 1999). Atypical antipsychotics improved side effects and efficacy have been attributed to the high affinity interaction with 5-HT_{2A}

receptors and lower affinity for D2 (Kasper, et al., 1999). Atypical antipsychotics have also been shown to block other 5-HT_2 receptor subtypes mainly 5-HT_{2B} and 5-HT_{2C} (Lucaites, et al., 1996;Zhang and Bymaster, 1999). However, only 5-HT_{2C} receptor antagonism is suggested in contributing to the atypical antipsychotic effects (Herrick-Davis, et al., 2000;Rauser, et al., 2001).

The 5-HT_{2A} receptor subtype has been implicated in various psychiatric disorders including depression, anxiety, and schizophrenia (Glennon, et al., 1984). Olanzapine is an atypical antipsychotic, approved for the treatment of schizophrenia and bipolar disorder. Olanzapine has been also studied in treatment of disorders like substance abuse, aggression/violence, borderline personality disorder, and obsessive–compulsive disorder (Littrell, et al., 2006).

Atypical antipsychotics as well as a specific 5-HT_{2A} receptor antagonist, MDL 100,907 ((+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol), desensitize 5-HT_{2A}-mediated responses (Willins, et al., 1999). However, the molecular mechanisms involved in antagonist-induced desensitization of 5-HT_{2A} receptor signaling are not well understood. By understanding the molecular mechanisms underlying the effects of olanzapine and other atypical antipsychotics, we hope to gain insight into targets for therapeutic treatment of psychiatric disorders. It has been recently reported that olanzapine increases Extracellular Receptor Kinase (ERK) 1/2 phosphorylation in rat prefrontal

cortex (Fumagalli, et al., 2006). Furthermore, changes in mRNA levels of both 5-$HT_{2A/2C}$ receptors with 5-HT_{2A} receptor antagonists after both chronic and short-term treatments have been reported (Buckland, et al., 1997). These studies suggest that atypical antipyschotics may target intracellular pathways shuttling information from the receptor to the nucleus.

The Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling cascade has been reported to couple with 5-HT_{2A} receptors in skeletal muscles and vascular smooth muscle cells (Guillet-Deniau, et al., 1997;Banes, et al., 2005). G-protein coupled receptor (GPCR) agonists, thrombin and angiotensin II, have previously been shown to activate JAK-STAT signaling cascade (Bhat, et al., 1994). JAK-STAT could be one of the possible signaling pathways involved in mediating olanzapine-induced receptor desensitization. Janus kinases (JAK) are a small family of cytoplasmic tyrosine kinases initially identified as a mediator of cytokine receptor signaling (Ihle, 1995). Agonist stimulation of cytokine receptors causes phosphorylation of JAK, which in turn phosphorylates tyrosine residues on the receptor cytoplasmic tail, facilitating activation of specific signal transducers and activators of transcription (STAT). Tyrosine phosphorylated STAT then undergoes dimerization and translocates to the nucleus where it binds to target DNA sequences (Darnell, Jr., 1997).

Alterations in proximal components of the 5-HT_{2A} receptor signaling system could mediate desensitization in response to increased activity of

intracellular cascades such as JAK-STAT. 5-HT_{2A} receptors are classically linked to the $G_{\alpha q/11}$ protein family (Ivins and Molinoff, 1990). Activation of $G_{\alpha q/11}$ stimulates phospholipase C (PLC) activity, which subsequently promotes the release of diacylglycerol and inositoltriphosphate, which in turn stimulate protein kinase C activity and calcium release (Berg, et al., 2001). It has been extensively reported that increased expression of regulators of G protein signaling (RGS) proteins cause desensitization of several G-protein associated receptor systems (Koelle and Horvitz, 1996). RGS proteins reduce the duration of signaling of many G-protein-coupled receptors by their action as GTPases, accelerating the hydrolysis of GTP bound G_{α} -proteins or by blocking the interaction of G_{α} with its target proteins through a not well-understood process known as effector antagonism (Roy, et al., 2006).

Expression of RGS7 protein in rat frontal cortex is well documented (Krumins, et al., 2004;Zhang and Simonds, 2000) and decreased 5-HT_{2A} receptor-mediated signaling via direct interaction of RGS7 protein with $G_{\alpha q}$ has been widely characterized in different systems (DiBello, et al., 1998;Ghavami, et al., 2004). We hypothesize that the increased expression of RGS7 protein by the JAK-STAT signaling cascade contributes to olanzapine-induced desensitization of 5-HT_{2A} receptor signaling. In this study, we examined 5-HT_{2A} receptor and RGS7 protein levels in response to treatment with olanzapine and determined whether changes in these proteins are mediated by olanzapine-induced JAK-STAT signaling in A1A1v cells.

Materials and Methods

Cell Culture:

A1A1v cells, a cortical cell line that expresses 5-HT_{2A} receptors, were used for all experiments and were generously donated by Dr. William Clarke and Kelly Berg (University of Texas Health Science Center, San Antonio, TX). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Serotonin was removed from serum by filtration after treating with charcoal. Cells were grown in the serotonin-free serum media 24 hours before treatment with olanzapine. Olanzapine was a generous gift from Eli-Lilly. The treatment concentration (300nM) was obtained by dissolving olanzapine in 20% acetic acid. The pH of the vehicle and olanzapine was adjusted to 6.5 with 10N NaOH. A JAK inhibitor, α-Cyano-(3,4-dihydroxy)-N-benzylcinnamide (AG490), was purchased from Calbiochem, San Diego, CA. It was reconstituted with DMSO to obtain the desired concentration. (-)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) was purchased from Sigma-Aldrich; St. Louis, MO. HBSS mix (1X HBSS, 20mM $LiCl_2$, and 20mM HEPES) was used to dissolve DOI. Cells were treated with either vehicle (20% acetic acid) or 300nM olanzapine for 24 hour for various experiments.

Cell fractionation:

Cells were separated into membrane and cytosol fractions using ultra centrifugation as previously described (Tucker, 2004). Briefly, cells were collected in hypotonic buffer containing 0.25M sucrose, 50mM Tris-HCl, 5mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO). Cell homogenate was prepared by sonication three times for 10 seconds. The homogenate was spun at 100,000xg for 45 minutes at 4^0C to produce a pellet, which is composed of membrane fraction, and a supernatant, which is the cytosol fraction. The pellet was reconstituted with hypotonic buffer. The protein amount was assessed with bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Cytosolic and nuclear fractions were prepared as previously described (Andrews and Faller, 1991) with some modification. Briefly, cells were washed and scrapped into ice-cold PBS containing phosphatase inhibitors. The pellet was collected after centrifugation at 1,000xg for one minute and resuspended into Buffer A (10mM HEPES-KOH pH 7.9, 1.5mM MgCl2, 10mM KCl, 0.5mM DTT, and protease inhibitor cocktail) and incubated for 10 minutes on ice followed by vortex mixing for 10 seconds. The supernatant contains the cytosolic fraction. The pellet was resuspended in Buffer B (10mM HEPES-KOH pH 7.9, 25% glycerol, 420mM NaCl, 0.2mM EDTA, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, and protease inhibitor cocktail) and incubated on ice for 20 min. The nuclear fraction was obtained as the supernatant after centrifugation at 18,000xg for 2 min.

Western Analyses:

Equal amounts of protein were separated and transferred to nitrocellulose membrane as described before (Berg, et al., 1994a). Membranes were blocked either in TBS or PBS containing 5% (w/v) nonfat dry milk with 0.1% Tween 20. The following primary antibodies were used: anti-RGS7 (1:1000; Polyclonal antibody, Upstate Biotechnology, Inc., Lake Placid, NY), anti-phospho-JAK2 (1:1000; polyclonal antibody, Affinity Bioreagent**,** CO), and anti-JAK2 (1:2000; polyclonal antibody, Upstate Biotechnology, Inc., Lake Placid, NY). The anti-phospho- STAT1, STAT3, STAT5 (1:1000; polyclonal antibodies), and anti-STAT1, STAT3, STAT5 (1:1000; polyclonal antibody) were purchased from Cell Signaling, (Danvers, MA). A monoclonal anti-actin antibody was from MP Biomedicals (1:10,000; Aurora, OH). Prior to incubation with a second primary antibody, blots were stripped with Restore western blot stripping buffer from Pierce (Rockford, IL) by incubating at 37^0C for 25 minutes. After incubation blots were removed from stripping buffer, washed three times for 10 min each with TBS or PBS containing 0.1% Tween20 and blocked again. Protein bands were analyzed densitometrically using Scion Image software (Scion Corporation, Frederick, MD). The IOD for the film background was subtracted from the IOD for each band. Each sample was measured in triplicate. RGS7 protein and 5-HT_{2A} receptor protein levels were normalized to actin protein used as a loading control and phosphoproteins were normalized to the corresponding total

protein levels. Protein levels from olanzapine-treated cells were normalized to vehicle-treated cells for each cell fraction.

5-HT_{2A} Receptor Antibody Production and Characterization:

A peptide corresponding to amino acids 22 to 41 of the rat 5-HT_{2A} receptor (NH2-GD PRLYHNDFNSRDANTSE-OH) was synthesized and used to produce antibodies by Biosynthesis, Inc. (Lewisville, TX). This sequence is 85% identical to the mouse 5-HT_{2A} receptor sequence and 65% identical to the human 5-HT_{2A} receptor sequence as determined using the NCBI Sequence Viewer. The same peptide sequence was used previously by Garlow et al. (Garlow, et al., 1993) to produce antibodies against the 5-HT_{2A} receptor. The antibodies produced were characterized using western blotting and ELISA assays. The antibody titer reported by Biosynthesis, Inc. was up to 1:25,600. A1A1v cells were used to verify the specificity of the 5-HT_{2A} receptor antibodies. Cells were transfected with the human 5-HT_{2A} receptor in pcDNA3.1+ (Guthrie DNA Resource Center, Sayre, PA) using 3μg of DNA for every dish and the Lipofectamine Plus Reagent (Invitrogen, Corp., Frederick, MD).

Inositol phosphate (IP) accumulation assay:

Assays were performed as previously described (Berg, et al., 1994). Briefly, cells were seeded in 24 well plates at the density of 40,000/well. Cells were treated with vehicle or 300nM olanzapine and also at the same time

labeled with 0.5 μCi [^{3}H] myoinositol/well for 24 hour in serum free DMEM media. Cells were washed with Hank's balanced salt solution containing 20 mM $LiCl_2$ and 20 mM HEPES, Ph 7.4. PI hydrolysis was initiated with the addition of DOI at 37^0C. Reaction was stopped after 30 min with ice-cold 10 mM formic acid. The accumulation of ^{3}H-labeled inositol phosphate (IP) was determined by ion exchange chromatography (Singh et al., 2007).

Statistics:

All statistical analyses were performed using GB-STAT School Pak (Dynamic Microsystems, Silver Spring, MD). Data are expressed as means ± SEM. For Western blots and the IP accumulation assay, data were analyzed using a Student's t test for equal variances.

Results

IP accumulation assay:

Agonist-stimulated IP accumulation can be used to monitor desensitization of 5-HT_{2A} receptor mediated signaling (Hanley and Hensler, 2002). Treatment with 300nM olanzapine for 24 hours significantly decreased ($p<0.05$) DOI (10^{-4} M) stimulated IP accumulation by 28±1.9% compared to vehicle (20% acetic acid) treated cells (figure 6). This decrease suggests a desensitization of 5-HT_{2A}-mediated receptor signaling by olanzapine. The DOI

concentration was chosen based on the previous dose-response experiments conducted in our laboratory (Shi, et al., 2007).

Olanzapine induced phosphorylation of JAK kinase:

Guillet-Deniau et al. (Guillet-Deniau, et al., 1997) have shown that serotonin stimulation of 5-HT_{2A} receptors causes phosphorylation of JAK2 kinase and association of the receptor with JAK2. To investigate whether olanzapine causes JAK2 phosphorylation, lysates of cytosol and membrane fractions prepared from vehicle (20% acetic acid) and olanzapine (300nM) treated cells were examined by western blot with anti-phospho-JAK2 antibody, then stripped and reprobed with anti-JAK2 antibody (figure 7A). Tyrosine phosphorylation of JAK2 was significantly increased ($p<0.05$) to more than 232 ±15 % of the control levels in the membrane fraction of olanzapine-treated cells (no significant change in cytosolic levels), whereas total JAK2 protein levels did not show any appreciable change.

Phosphorylation and nuclear translocation of STAT Proteins:

Phosphorylated JAK2 facilitates activation of various STAT proteins. Tyrosine phosphorylated STATs then undergoes dimerization, translocate to the nucleus and bind to the target DNA sequences. However, different STAT proteins mediate signaling cascades stimulated by different agonists. To determine which STAT protein(s) are activated in response to olanzapine,

lysates of cytosolic and nuclear fractions from control (20% acetic acid) and olanzapine (300nM) treated cells were analyzed by western blot with anti-phospho- STAT1, STAT3, and STAT5 antibodies, then stripped and reprobed with corresponding anti-STAT antibodies. We found that phosphorylation and nuclear translocation STAT3 was significantly increased to 171±25% in the olanzapine-treated cells compared to vehicle-treated cells (figure 7B), where as phosphorylation and nuclear translocation of STAT1 and STAT5 did not show any change (data not shown).

Olanzapine increases RGS7 protein levels:

In order to monitor the changes in RGS7 protein levels, lysates from cytosol and membrane fractions of vehicle (20% acetic acid) and olanzapine (300nM) treated cells were analyzed by western blot with anti-RGS7 antibody (figure 8A). We found that RGS7 protein levels were increased in the membrane fraction to 160±18% of control levels and significantly increased in cytoplasmic fraction by 193±32% of control levels in olanzapine-treated cells compared to vehicle-treated control cells.

Characterization of 5-HT_{2A} receptor antibody:

A1A1v cells were used to verify the specificity of the 5-HT_{2A} receptor antibody. On western blots prepared with A1A1v cell lysates, there was a prominent band with a molecular mass of approximately 42 kilodaltons (figure

3B). This is very similar to the size of the band produced by *in vitro* transcription and translation of the human 5-HT_{2A} receptor construct provided by Guthrie cDNA Resource Center (Sayre, PA). Over expression of the human 5-HT_{2A} receptor construct in A1A1v cells resulted in a more intense protein band detected on western blots prepared with the 5-HT_{2A} receptor antibody (figure 8B). Preadsorption control experiments were also performed to verify the specificity of the antibody. Using homogenates from rat frontal cortex and lysates from A1A1v cells transfected with the human 5-HT_{2A} receptor, the 42 kilodalton band 5-HT_{2A} receptor band was no longer present in the western blots prepared with the antibody pre-incubated with the peptide antigen (figure 8C). These experiments also demonstrate that the antibody produced using a peptide based on the sequence for rat 5-HT_{2A} receptor, cross-reacts with the human 5-HT_{2A} receptor expressed in rat cells. Furthermore, the 5-HT_{2A} receptor antibody also cross-reacts with the rat 5-HT_{2A} receptor expressed in a rat cortical cell line.

5-HT_{2A} receptor protein levels:

Cytosol and membrane fractions from vehicle (20% acetic acid) and olanzapine (300nM) treated cells were analyzed by western blot with the anti-5-HT_{2A} antibody we generated. We found a significant increase ($p<0.05$) in both cytoplasmic (234±32% of control level) and membrane fractions (206±14% of control levels) of olanzapine-treated cells compared to vehicle-

treated cells (figure 8D). We also assessed the purity of our membrane fraction after stripping and reprobing the same blot with an anti-NA+-K+-ATPase antibody. A band corresponding to NA+-K+-ATPase was mainly present in membrane fraction (data not shown).

JAK inhibition:

A JAK kinase inhibitor, AG490, was used to investigate whether inhibition of the JAK-STAT signaling cascade could reverse the increase of 5-HT_{2A} receptor or RGS7 protein levels observed in response to olanzapine treatment. Cells were treated for one hour with 0, 15, and 30 μM of AG 490, before adding either vehicle or olanzapine. Twenty-four hours later, cells were lysed and protein levels of phospho-JAK2, RGS7, and phospho-STAT3 were analyzed by western blot. Olanzapine-induced phosphorylation of JAK2 was decreased with AG490 as shown in figure 9A. There was no change in the total JAK2 protein levels. With AG490 treatment, there was a similar decrease in olanzapine-induced STAT3 phosphorylation in the nuclear fraction as shown in figure 9B, and no change in STAT levels, again confirming previous findings that activation of JAK2 causes phosphorylation and nuclear localization of STAT3.

If the JAK-STAT signaling cascade is mediating the olanzapine-induced increase in protein levels, then inhibiting this signaling cascade should prevent the increase in 5-HT_{2A} receptor and RGS7 protein levels. To

test this hypothesis, membrane fractions from vehicle- and olanzapine-treated cells, pretreated with AG490 were analyzed by western blot for 5-HT_{2A} receptor and RGS7 protein levels. As in previous experiment (figure 8A) olanzapine treatment for 24 hours increased the levels of RGS7 protein (figure 10A and B). Treatment with AG490 reduced the olanzapine-induced increase in the RGS7 in the membrane fractions to the levels in the vehicle treated cells (figure 10A). Treatment with AG490 alone had no effect on the levels of RGS7 protein. Although we observed a similar increase in levels of 5-HT_{2A} receptor protein in olanzapine-treated cells compared to vehicle-treated cells as shown before in figure 8C, AG490 pretreatment did not alter protein levels of 5-HT_{2A} receptor in cells treated with olanzapine (figure 10C and D) suggesting that the increase in levels are not mediated by JAK-STAT signaling cascade.

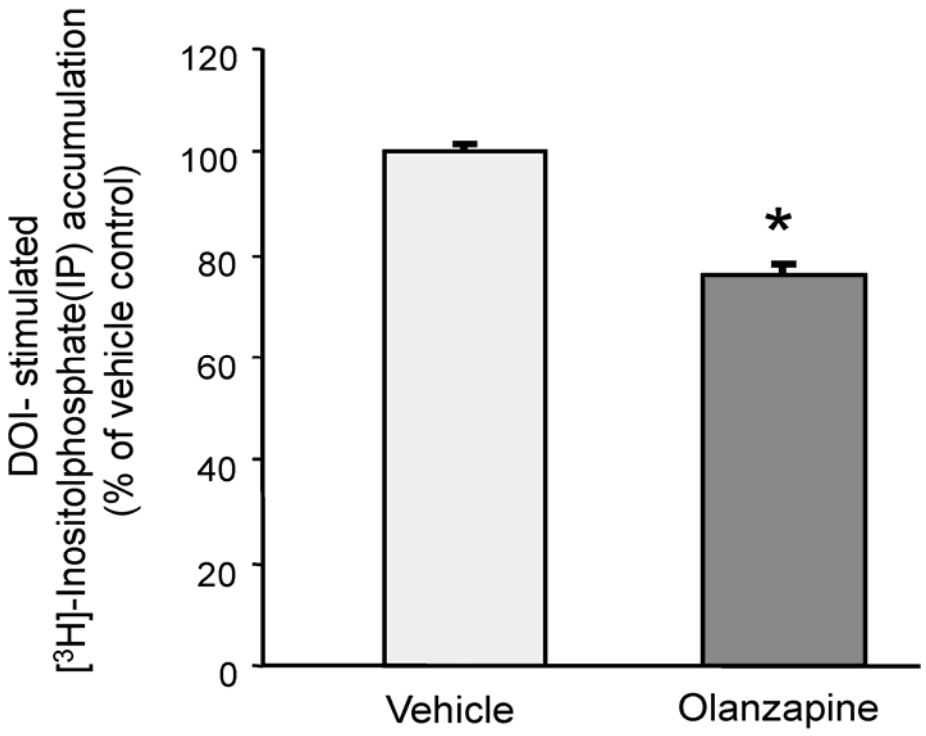

Figure 6. Olanzapine decreases DOI-stimulated IP accumulation. A1A1v cells were treated either with vehicle (20% acetic acid) or olanzapine (300nM) for 24 hours, and incubated with [3H] myoinositol for same 24-hour period. Both vehicle- and olanzapine-treated cells were stimulated with 10^{-4} M DOI. Bar graph represents the mean DOI-stimulated IP accumulation normalized to DOI-stimulated IP accumulation in vehicle-treated cells from three independent experiments. IP accumulation was significantly decreased in olanzapine-treated cells compared to vehicle-treated cells. * indicates significantly different from vehicle-treated cells at $p < 0.05$.

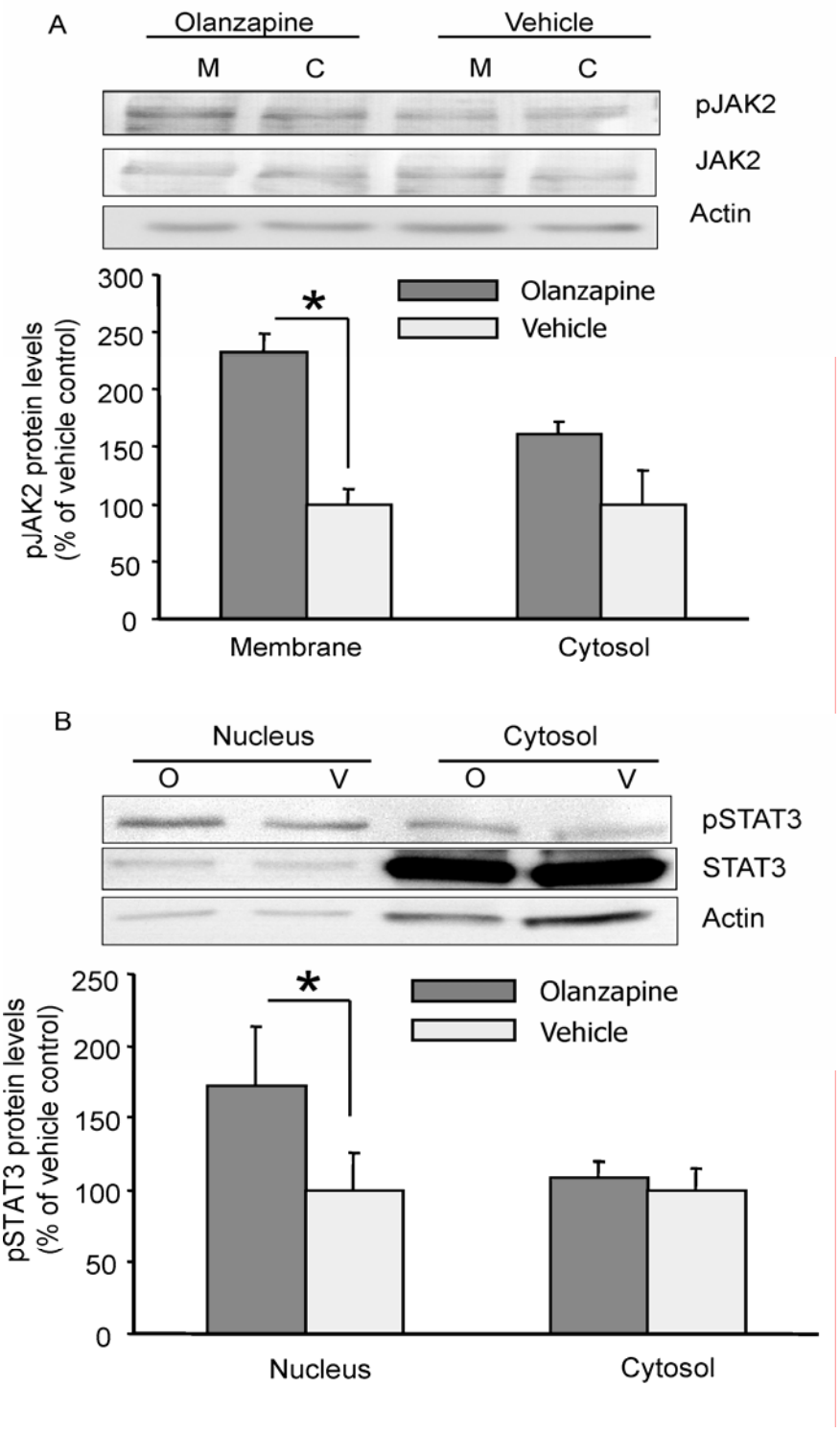

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Figure 7. Olanzapine activates the JAK-STAT signaling cascade. A1A1v cells were treated either with vehicle (20% acetic acid) or (300nM) olanzapine for 24 hour. (A) Cytosol (C) and membrane (M) fractions of cells were analyzed by western blot with an anti-phosphoJAK2 antibody, stripped and reprobed with anti-JAK2 and anti-actin antibodies. Bar graph represents quantification of phosphoJAK2 protein levels divided by JAK2 protein levels from three independent experiments. Phosphorylation of JAK2 was significantly (*$p<0.05$) increased with olanzapine-treatment compared to vehicle-treated cells. (B) Cytosol (C) and nuclear fractions (N) of cells were analyzed by western blot with anti-phosphoSTAT3 antibody, stripped and reprobed with anti-STAT3 and anti-actin antibodies. Bar graph represents quantification of phosphoSTAT3 protein levels divided by STAT3 protein levels from three independent experiments. Olanzapine treatment significantly increases the phospho-STAT3 in the nucleus compared to vehicle treatment. * indicates significantly different from vehicle-treated cells at $p < 0.05$.

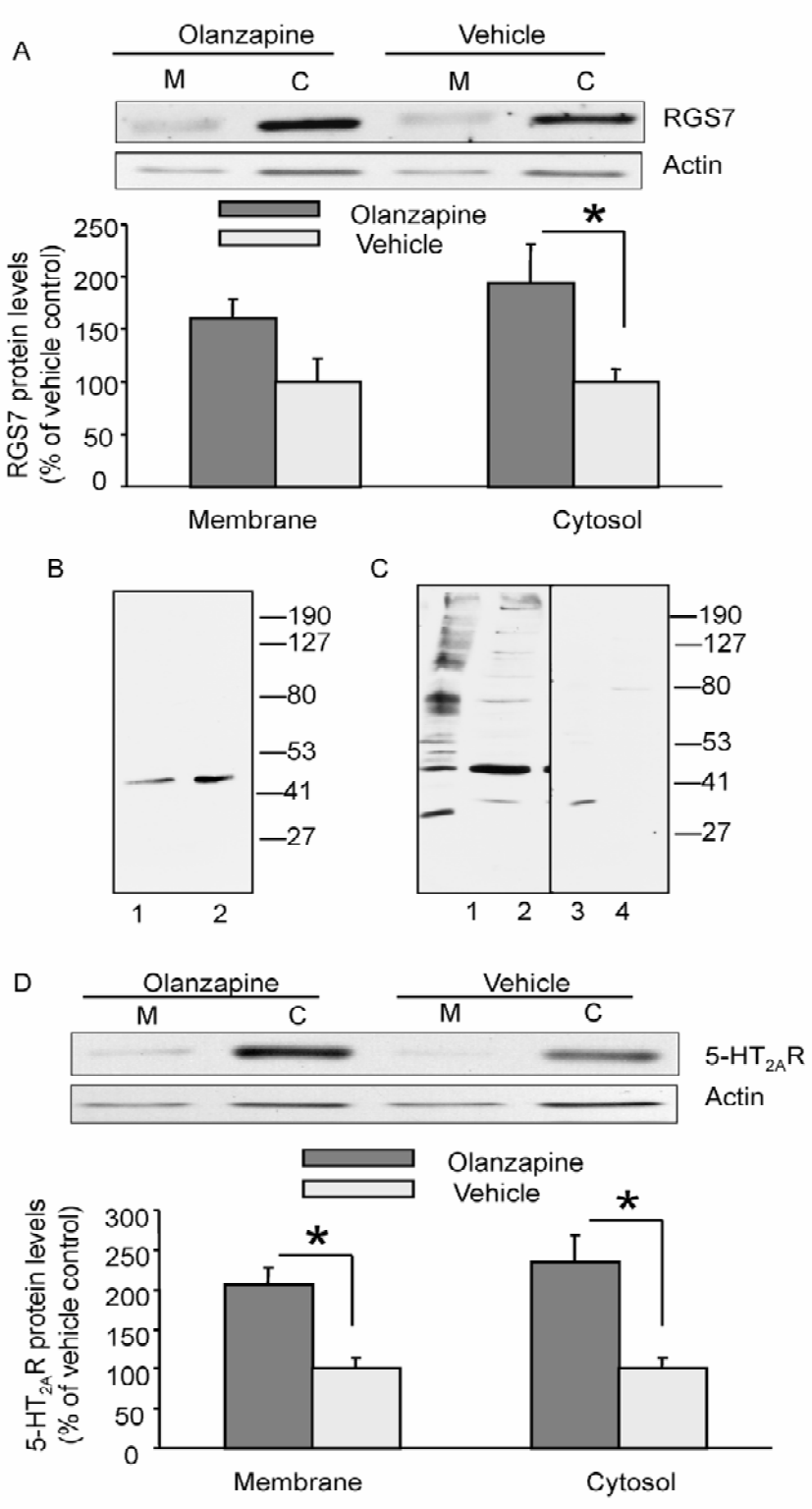

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Figure 8. Olanzapine treatment increases the levels of RGS7 protein and 5-HT_{2A} receptor (5-HT_{2A}R) protein levels. (A) A1A1v cells were treated with either vehicle (20% acetic acid) or 300nM olanzapine for 24 hour. Cytosol (C) and membrane (M) fractions of cells were analyzed by western blot with anti-RGS7 antibody, stripped and reprobed with an anti-actin antibody as a loading control. Bar graph represents quantification of RGS7 protein levels divided by actin protein levels from three independent experiments. Protein levels of RGS7 were significantly (*$p<0.05$) increased in olanzapine-treated cells compared to vehicle-treated cells. (B) A1A1v cells transfected with human 5-HT_{2A} receptor (lane 2) produced a larger band than untransfected cells (lane 1) probed by 5-HT_{2A} receptor antibody. (C) Preabsorbtion of the 5-HT_{2A} receptor antibody blocked the production of the 42kd protein band for 5-HT_{2A} receptor. Western blots prepared with homogenates of rat frontal cortex (lanes 1 and 3) and with lysates of A1A1v cells transfected with human 5-HT_{2A} receptor (lanes 2 and 4) were incubated with 5-HT_{2A} receptor antibody (lanes 1 and 2) or 5-HT_{2A} receptor antibody preadsorbed with the peptide antigen (lanes 3 and 4). (D) A1A1v cells were treated with either vehicle (20% acetic acid) or 300nM olanzapine for 24 hour. Cytosol and membrane fractions of cells were analyzed by western blot with anti- 5-HT_{2A} antibody, stripped and reprobed with anti-actin antibody as a loading control. Bar graph represents quantification of 5-HT_{2A} protein levels divided by actin protein levels from four independent experiments. 5-HT_{2A} protein levels in both

cytosol and membrane fractions were significantly increased in olanzapine-treated cells compared to vehicle-treated cells. * indicates significantly different from vehicle-treated cells at $p < 0.05$.

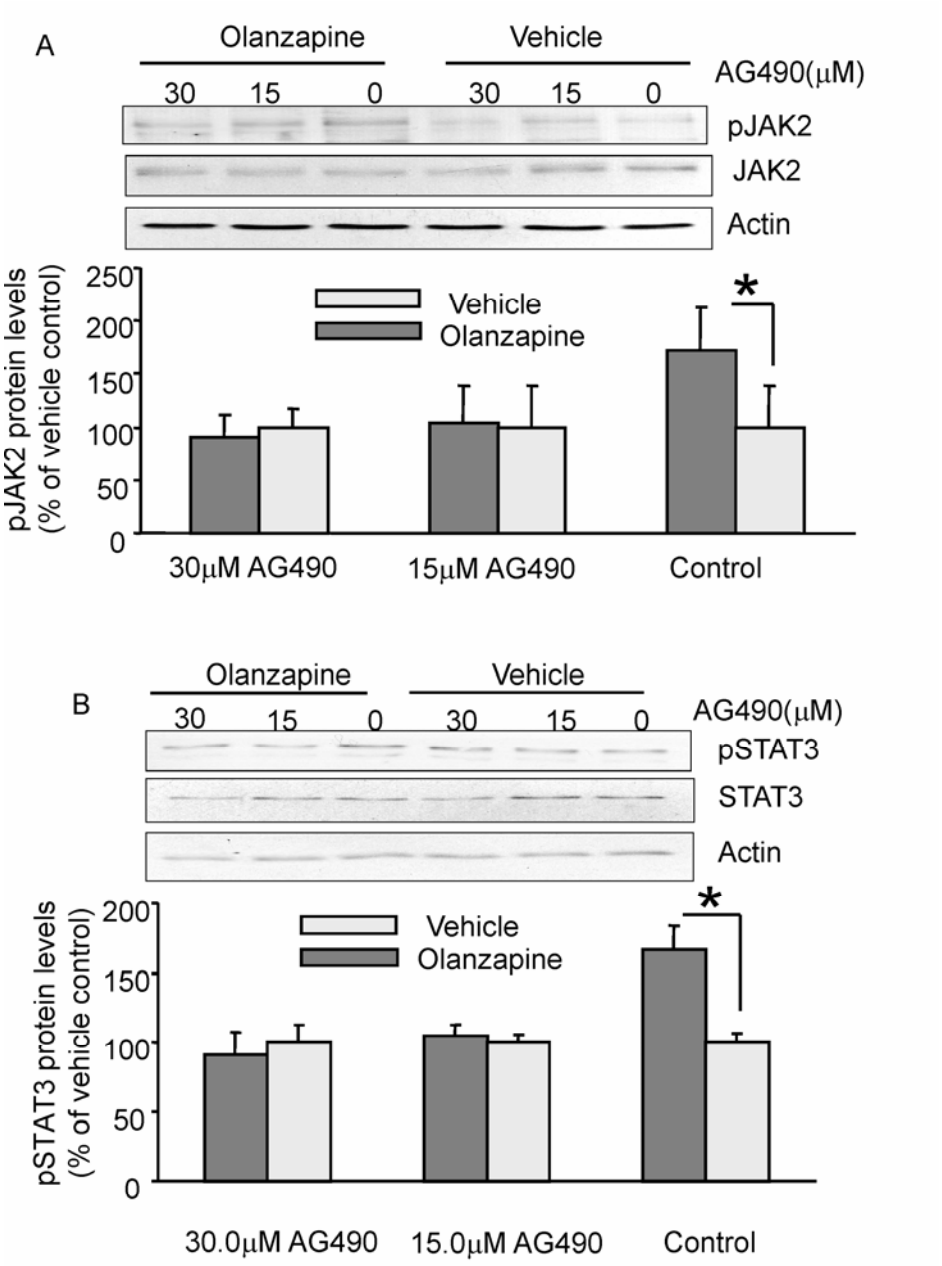

Figure 9. AG490 pretreatment inhibited olanzapine-induced increases in JAK-STAT signaling. A1A1v cells were pretreated for 1hour with the indicated concentrations of AG490 prior to treating with either vehicle (20% acetic acid)

or 300nM olanzapine for 24 hours. (A) Membrane fraction of cells was analyzed by western blot with anti-phosphoJAK2 antibody. Blots were stripped and reprobed with anti-JAK2 and anti-actin antibodies. Bar graph represents quantification of phosphoJAK2 protein levels divided by JAK2 protein levels from three independent experiments. (B) Nuclear fractions were analyzed by western blot with anti-phosphoSTAT3 antibodies, stripped and reprobed with anti-STAT3 and anti-actin antibodies. Bar graph represents quantification of phospho-STAT3 protein levels divided by STAT3 protein levels from three independent experiments. AG490 pretreatment decreases phosphoJAK2 levels and phosphoSTAT3 nuclear translocation in olanzapine treated cells compared to cells treated olanzapine and the AG490 vehicle. * indicates significantly different from vehicle-treated cells at $p < 0.05$.

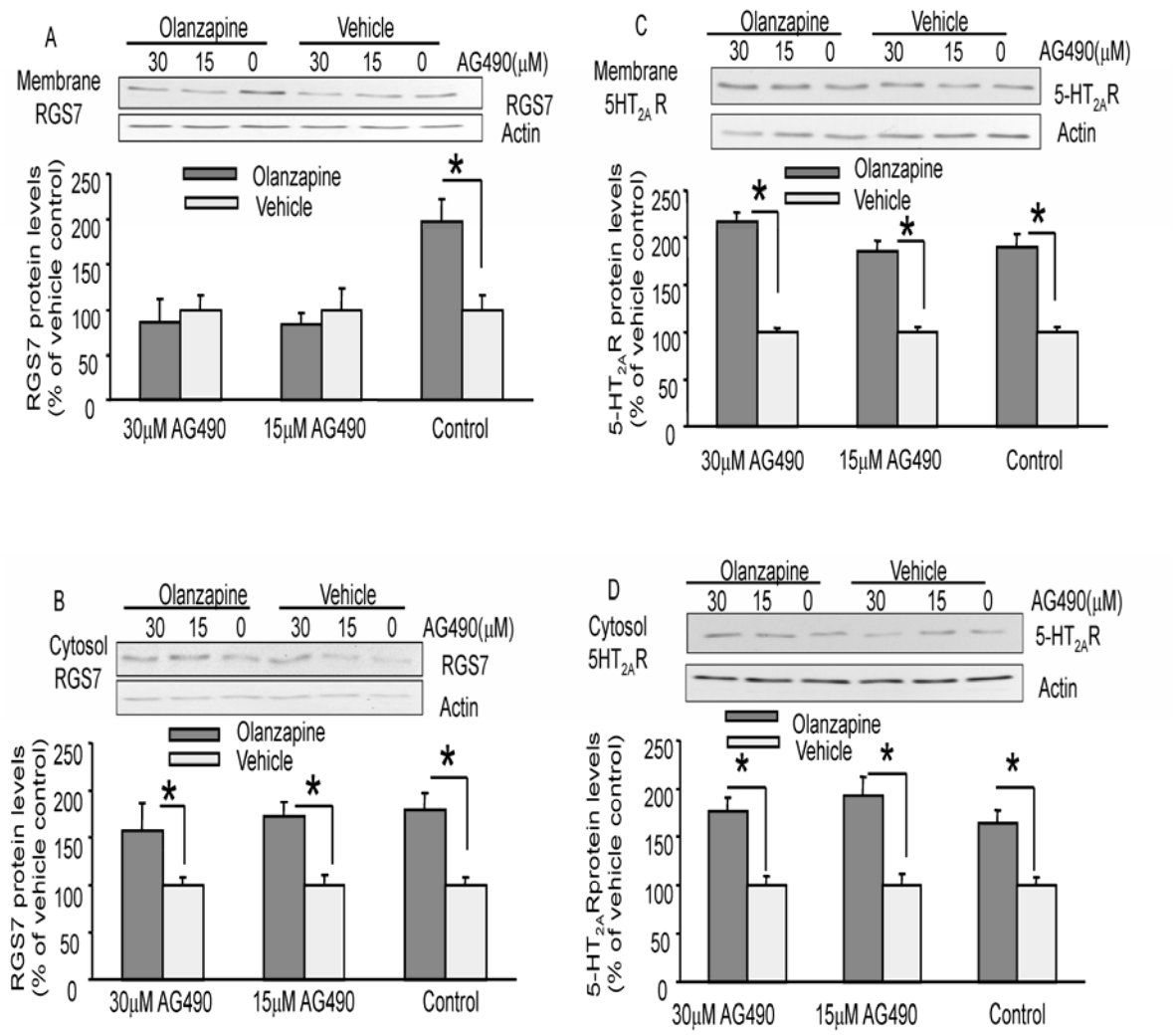

Figure 10. AG490 pretreatment reduces the olanzapine-induced increase in RGS7 protein levels in membrane but not the levels of $5HT_{2A}$ receptor protein ($5\text{-}HT_{2A}R$). A1A1v cells were pretreated with the indicated concentrations of AG490 for 1hour, before treating with either vehicle (20% acetic acid) or 300nM olanzapine for 24 hours. (A) The membrane and (B) cytosol fraction of

cells were analyzed by western blot with anti-RGS7 antibody, stripped and reprobed with anti-actin antibody; actin was used as a loading control. Bar graph represents quantification of RGS7 protein levels divided by actin protein levels from three independent experiments. (C) Membrane and (D) cytosol fractions of cells were analyzed by western blot with anti-5-HT_{2A} receptor antibody, stripped and reprobed with anti-actin antibody as a loading control. Pretreatment with AG490 had no effect on the increase in 5-HT_{2A} receptor protein levels induced by olanzapine. * indicates significantly different from vehicle-treated cells at $p < 0.05$.

Discussion

This study demonstrates the involvement of an intracellular signaling cascade, the JAK-STAT pathway, in increasing the levels of RGS7 protein in response to treatment with olanzapine. The increased levels of RGS7 protein in turn could contribute to the desensitization of 5-HT_{2A} receptor signaling induced by olanzapine in A1A1v cells by directly interacting with $G_{\alpha q/11}$ and accelerating GTP hydrolysis. In contrast, we found an increase in 5-HT_{2A} receptor protein levels with olanzapine treatment that was not associated with increased activation of the JAK-STAT signaling cascade. The increase in 5-HT_{2A} receptor protein levels would not likely contribute to the desensitization response but could conceivably counter or moderate the desensitization response.

Olanzapine and other antipsychotics have been recently reported to stimulate other signaling cascades including increased phosphorylation of ERK1/2 in rat frontal cortex (Fumagalli, et al., 2006), and increased phosphorylation of Akt/PKB and p38 in PC12 cells (Lu, et al., 2004). The increased activation of signaling cascades induced by antipsychotics suggests that changes in gene expression regulated by these cascades could contribute to the positive therapeutic benefits seen in schizophrenic patients.

Several micro-array studies have explored the effects of olanzapine and other antipsychotics on gene expression, reporting changes in expression of a host of genes including RGS proteins, and gene families

linked with synaptic plasticity and presynaptic neurotransmission (Feher, et al., 2005;Fatemi, et al., 2006). Fatemi et al. (Fatemi, et al., 2006), reported an increase in expression of RGS19 mRNA and a decrease in expression of RGS2 mRNA in the frontal cortex of rats treated for three weeks with olanzapine. GAP activity of RGS19 is associated with $G_{\alpha z}$, a member of $G\alpha i/o$-G protein family, whereas 5-HT_{2A} receptors are coupled with $G_{\alpha q/11}$ proteins. Therefore, increased RGS19 is unlikely to affect olanzapine-induced desensitization of 5-HT_{2A} receptor signaling. Although RGS2 protein associates with $G_{\alpha q}$ protein, and could decrease the 5-HT_{2A} receptor signaling; a decrease in expression of RGS2 protein could not cause the desensitization of 5-HT_{2A} receptor signaling induced by olanzapine since a decrease in RGS protein expression would likely result in increased receptor signaling. Although RGS2 is expressed in frontal cortex, it is not known if it co-localizes with 5-HT_{2A} receptors in frontal cortex.

None of these microarray studies identified alterations in RGS7 protein or 5-HT_{2A} receptor mRNA levels. It is important to remember that changes in mRNA levels may or may not result in changes in protein levels and changes in protein levels may not be due to changes in mRNA levels. It is also important to emphasize that these studies were conducted in different experimental settings with different time course and dose regimens than our study. Each study highlights important findings, further extending our understanding of antipsychotics mechanism of action. It is becoming evident

that there could be numerous pathways and alterations in gene expression that lead to the development of psychosis (Ko, et al., 2006) and its treatment with atypical antipsychotics.

Direct association of JAK2 and STAT3 with 5-HT_{2A} receptors, and activation of the JAK-STAT signaling cascade by 5-HT_{2A} receptor agonists has been reported previously (Guillet-Deniau, et al., 1997). In the present study, we found JAK2 activation and increased phosphorylation and nuclear localization of phospho-STAT3 in olanzapine-treated cells. It is interesting to note that phosphorylation and nuclear translocation of other isoforms of STAT proteins, STAT1 and STAT5, did not show any change with olanzapine treatment. Activation of STAT proteins, which are transcription factors, could bolster the previous notion that antipsychotic agents affect expression of various genes.

Our experiments with a JAK kinase inhibitor, AG490, suggest that the increased levels of RGS7 protein are mediated by the JAK-STAT signaling cascade in response to olanzapine treatment. Interestingly, AG490 selectively targeted the membrane localized RGS7 protein and had no effect on the levels in cytosol fraction. The overall effect of AG490 is a decrease in the total levels of RGS7 protein (i.e., membrane plus cytosol), suggesting that olanzapine-induced JAK-STAT signaling increases the levels of RGS7 protein rather than causing a redistribution of the protein. We speculate that transcriptional activity of phospho-STAT3 could increase RGS7 mRNA

expression and be responsible for increased levels of RGS7 protein in the membrane fraction. The mechanism(s) involved in the selective decrease in the membrane fraction of A1A1v cells are unknown but could involve a chaperone protein. However, further studies are needed to test these hypotheses. In contrast, changes in 5-HT_{2A} receptor protein levels seem to be regulated by a different mechanism, since pretreatment with AG490 fails to reverse the olanzapine-induce increase in receptor protein levels.

Most atypical antipsychotics cause desensitization of 5-HT_{2A} receptor signaling. The decrease in DOI stimulated IP accumulation with olanzapine treatment in our study is consistent with these findings and supports the use of A1A1v cells as a model to study the actions of olanzapine on 5-HT_{2A} receptor signaling. Previous experiments in A1A1v cells using the selective 5-HT_{2A} receptor antagonist, MDL100907 demonstrated that IP accumulation stimulated by the 5-$HT_{2A/2C}$ selective agonist DOI is not likely due to stimulation of 5-HT_{2C} receptors (Shi, et al., 2007). Numerous investigations have explored the mechanisms by which antipsychotics cause desensitization of 5-HT_{2A} receptor signaling. This desensitization by antipsychotics could be mediated by receptor internalization, uncoupling of G-proteins from receptor, and receptor down-regulation (Roth, et al., 1995;Willins, et al., 1999) in addition to increases in RGS7 protein.

Other mechanisms have been associated with desensitization of 5-HT_{2A} receptor signaling as a result of treatment with atypical antipsychotics.

Several studies specifically investigated changes in the transcript level of 5-HT_{2A} receptors with both short term and chronic antipsychotic treatments (Doat-Meyerhoefer, et al., 2005;Burnet, et al., 1996). Buckland et al.(Buckland, et al., 1997) reported a significant decrease in receptor mRNA in hippocampus, brain stem and midbrain whereas no significant change was observed in other brain regions after 32 days of treatment with the atypical antipsychotic clozapine. However, in the same study, four days of clozapine treatment did not produce any significant change but a trend for a decreased mRNA expression was observed in major brain areas. As noted previously, changes in mRNA levels do not necessarily result in corresponding changes in protein levels, so changes in 5-HT_{2A} receptor mRNA levels are not necessarily inconsistent with the current findings. We found an increase in 5-HT_{2A} receptor protein levels in both the cytosol and membrane fractions. Previous studies reported a sizeable amount of 5-HT_{2A} receptors in the cytosol in addition to that found in the membrane (Cornea-Hebert, et al., 1999). Several reports suggest a decrease in Bmax with no change in Kd after treatment with antipsychotics (Doat-Meyerhoefer, et al., 2005;Matsubara and Meltzer, 1989). A decrease in the density of 5-HT_{2A} receptors without any change in affinity is consistent with the receptor internalization previously reported (Roth, et al., 1995;Willins, et al., 1998). An increase in 5-HT_{2A} receptor protein levels in the cytosol as we found with western blot analysis, could reflect internalized receptors. Furthermore, an increase in total 5-HT_{2A}

receptor protein levels is not necessarily inconsistent with a decrease in receptor density. 5-HT_{2A} receptor proteins undergo extensive post-translational modifications that could affect ligand binding. It is difficult to speculate at this point about the post-translational modifications that are required for or inhibit ligand binding. It is likely that multiple mechanisms contribute to desensitization of 5-HT_{2A} receptor signaling and that the specific mechanisms involved are likely tissue specific.

In summary, our cell culture data highlight a new role of JAK-STAT signaling in treatment with olanzapine. Increased-activation of this pathway by olanzapine increases expression of RGS7 protein. Increased RGS7 protein could directly contribute to the desensitization of 5-HT_{2A} receptor signaling by accelerating hydrolysis of GTP bound Gαq protein. Unfortunately, the JAK inhibitor AG490 interferes with the IP accumulation assay and therefore precludes our ability to determine if JAK-STAT signaling contributes to the desensitization of 5-HT_{2A} receptors in our model system. We are further investigating whether increased RGS7 protein is the result of increased transcriptional activity and whether blocking membrane localization of RGS7 protein inhibits desensitization of 5-HT_{2A} receptor signaling cascade. Further studies are also needed in animal models to confirm these findings *in vivo*. Overall, the results from this study provide a further understanding of possible involvement of intracellular pathways in mediating the effects of atypical antipsychotics.

CHAPTER IV

THE ROLE OF THE JAK-STAT PATHWAY IN ATYPICAL ANTIPSYCHOTIC-INDUCED DESENSITIZATION OF 5-HT_{2A} RECEPTOR SIGNALING IN CELL CULTURE

Abstract

We previously demonstrated that olanzapine-induced desensitization of 5-HT_{2A} receptor signaling is associated with increases in RGS7 protein levels both in vivo and in cells in culture, and the increase in RGS7 is dependent on activation of the JAK-STAT pathway in cells in culture (Muma, et al., 2007;Singh, et al., 2007). In the current study, we found that desensitization of 5-HT_{2A} receptor signaling induced by olanzapine is dependent on activation of the JAK-STAT pathway. Similar to olanzapine, clozapine-induced desensitization of 5-HT_{2A} receptor signaling is accompanied by increases in RGS7 (194±11% of control levels) and activation of JAK2 (196±18% of control levels). Treatment with the selective 5-HT_{2A} receptor antagonist MDL100907 for 24 h also increased RGS7 protein levels (176 ±16 % of control levels) and JAK2 activation (183 ±13 % of control levels). Olanzapine, clozapine, and MDL100907 treatment for 24 h increased mRNA levels of RGS7. Olanzapine treatment for 24 h increased STAT3 binding to the putative RGS7 promoter region. Taken together, olanzapine-induced activation of the JAK-STAT pathway, and increases in STAT3

binding to the RGS7 gene could underlie the increase in RGS7 mRNA which could subsequently increase protein expression. Furthermore, the increase in RGS7 protein could play a role in the desensitization of 5-HT_{2A} receptor signaling by terminating the activated $G_{\alpha q/11}$ proteins more rapidly. Overall, our data suggest that the full desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics requires activation of JAK-STAT pathway, which in turn increases RGS7 expression likely by direct transcriptional activity of STAT3.

Introduction

Atypical antipsychotics like clozapine and olanzapine (dibenzopyridine derivatives) represent a relatively new generation of antipsychotics with fewer incidences of negative side effects such as extrapyramidal side effects (EPS) (Meltzer, 1995). Although, atypical antipsychotics have a diverse receptor binding profile, 5-HT-receptor-based mechanisms have been postulated to play a critical role in the action of the atypical antipsychotic drugs (Willins, et al., 1999a). However, the process by which these drug-receptor interactions translate into long-term cellular adaptive changes resulting in antipsychotic efficacy is unknown.

Atypical antipsychotic drugs bind with high affinity to 5-HT_{2A} receptors and desensitize 5-HT_{2A} receptor signaling (Deutch, et al., 1991;Meltzer and Nash, 1991;Meltzer and Nash, 1991;Seeger, et al., 1995). Although,

desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics is reported to be associated with down-regulation and internalization (Willins, et al., 1999); the molecular mechanisms that underlie these changes are not well understood. Activation of 5-HT_{2A} receptors stimulates activation of $G_{\alpha q/11}$, which in turn activates effector enzymes including phospholipase C (PLC). PLC catalyses release of diacylglycerol (DAG) and inositoltriphosphate (IP3) from phosphatidyl inositol bisphosphate (PIP2). The released IP3 can be measured as an index of 5-HT_{2A} receptor signaling activity. In addition to these integral components of receptor signaling system, regulators of G protein signaling (RGS) proteins modulate signaling of several G protein coupled receptors (GPCR) (Koelle and Horvitz, 1996). RGS proteins can regulate G-protein signaling by functioning as GTPase-activating proteins (GAPs). GAP activity can hasten the termination of a signal upon removal of a stimulus, attenuate a signal either as a feedback inhibitor or in response to a second input, promote regulatory association of other proteins, or redirect signaling within a G protein signaling network (Ross and Wilkie, 2000). RGS4 and RGS7 are highly enriched in various brain regions including frontal cortex and are reported to be GAPs for $G_{\alpha q/11}$ associated 5-HT_{2A} receptor signaling (Larminie, et al., 2004). Khawaja et al. (1999) have extensively characterized cellular co-localization of RGS7 with $G_{\alpha q/11}$ immunohistochemically throughout the adult rat brain and reported a heterogeneous and overlapping regional distribution (Khawaja, et al., 1999).

We have previously reported that desensitization of 5-HT_{2A} receptor signaling with chronic treatment of olanzapine is accompanied by activation of STAT3 and an increase in RGS7 protein levels in rat frontal cortex (Muma, et al., 2007). In addition, we found that 24-h treatment with olanzapine causes desensitization of 5-HT_{2A} receptor signaling and an increase in membrane-associated RGS7 protein that is dependent on activation of the JAK2-STAT3 pathway in A1A1v cells, a cell line endogenously expressing the 5-HT_{2A} receptor signaling components (Singh, et al., 2007). However, whether activation of JAK-STAT is necessary for olanzapine induced desensitization and the mechanisms by which activation of the JAK-STAT pathway increase RGS7 protein are not currently known. Therefore, it is important to determine not only the role of the JAK-STAT pathway but also the mechanisms underlying up-regulation of RGS7 protein in response to antipsychotic treatment to help identify new targets for therapeutic intervention.

Increases in RGS7 protein levels could be mediated by several mechanisms for example, RGS7 binding to $G_{\beta 5}$ is reported to increase stability of each protein (Chen, et al., 2003) such that an increase in $G_{\beta 5}$ could increase RGS7 protein levels. Another possible mechanism is a direct increase in transcription of RGS7 thereby increasing RGS7 mRNA levels. We previously reported that inhibition of activated JAK-STAT pathway, completely blocked the increase in RGS7 protein levels by olanzapine (Singh, et al., 2007). Although, transcriptional activity of STAT3 as been extensively

reported for various genes (Aaronson and Horvath, 2002;Kisseleva, et al., 2002;Schindler, 2002), STAT3 has not been identified as a transcription factor for RGS7. STAT3-mediated regulation of gene expression is associated with the presence of the consensus element $TTCN_{2-4}GAA$ upstream of the transcription start site (Ehret, et al., 2001;Wrighting and Andrews, 2006). Genomic sequence analysis of rat RGS7 revealed that there are multiple sets of $TTCN_{2-4}GAA$ sequences. Thus, it is possible that STAT3 is a transcription factor for the RGS7 promoter.

Based on our previous reports that the olanzapine-induced increases in RGS7 protein levels are dependent on activation of the JAK-STAT pathway, we hypothesize that STAT3 is a transcription factor for RGS7 and is directly responsible for the increase in RGS7 protein levels by olanzapine treatment. In this study, we also examined whether another atypical antipsychotic, clozapine and a selective 5-HT_{2A} receptor antagonist, MDL100907, also activate the JAK-STAT pathway and increase RGS7 expression. Lastly, we determined whether activation of JAK-STAT pathway is necessary for desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics.

Materials and Methods

Drugs:

Olanzapine and AG490 were purchased from Torrent Research Chemicals Inc., ON, Canada. MDL100907 was kindly provided by Sanofi

Aventis, Bridgewater, NJ. Clozapine was purchased from TOCRIS, Ellisville, MO. Olanzapine was dissolved in 20% glacial acetic acid and the pH was adjusted to 6.5 with 10M NaOH as described in previous chapter. AG490, MDL100907 and clozapine were dissolved in 100% DMSO to obtain the desired concentration for individual treatments with each drug. (-)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) was purchased from Sigma-Aldrich; St. Louis, MO. HBSS mix (1X HBSS, 20mM $LiCl_2$, and 20mM HEPES) was used to dissolve DOI. A stock solution of 100μM clozapine was prepared in DMSO. For clozapine treatment each group was added equal volume (10μl) in 10 ml of cell culture media of either drug or vehicle (equal volume of DMSO was added to each group).

Cell Culture:

A1A1 cells, a cortical cell line, that endogenously expresses 5-HT_{2A} receptors and its downstream components, were used for all experiments (generously donated by Dr. William Clarke and Kelly Berg, University of Texas Health Science Center, San Antonio, TX). Cells were grown in the charcoal-treated serum to diminish serotonin in the media 24 h before treatment with olanzapine, clozapine, or MDL100907. Cells were treated with either vehicle or drugs for 24 h.

Cell fractionation:

Cell lysates were separated into membrane and cytosol fractions using centrifugation as previously described (Tucker, 2004;Singh, et al., 2007). Briefly, cells were washed once with PBS containing phosphatase inhibitors, followed by incubation in hypotonic buffer containing 0.25M sucrose, 50mM Tris-HCl, 5mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO) for 15 min on ice before lifting them with a cell scrapper. Cells were spun at 500xg at 4^0C to remove cell debris and then sonicated three times for 10 sec to make a cell homogenate. The homogenate was spun at 100,000xg for 45 min at 4^0C to produce a pellet, which is composed of membrane fraction, and a supernatant, which is the cytosol fraction. The pellet was reconstituted with hypotonic buffer. The protein concentration was assessed with bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

Western Analyses:

Equal amounts of protein from vehicle-control and drug-treated samples were separated on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membrane for 2 h at 100V. Non-specific binding to the membranes was blocked either with TBS containing 5% (w/v) nonfat dry milk with 0.1% Tween 20 (TBST) or in PBS containing 5% (w/v) nonfat dry milk. The following primary antibodies were used: anti-RGS7 (Polyclonal

antibody, Upstate Biotechnology, Inc., Lake Placid, NY), anti-phospho-JAK2 (polyclonal antibody, Affinity Bioreagent, CO), anti-JAK2 (polyclonal antibody, Upstate Biotechnology, Inc., Lake Placid, NY), and anti-actin (monoclonal antibody MP Biomedicals, Aurora, OH). Prior to incubation with a second primary antibody, blots were stripped with Restore western blot stripping buffer (Pierce, Rockford, IL) by incubating at 37^0C for 25 min. After incubation, blots were removed from stripping buffer, washed three times for 10 min each with TBS or PBS containing 0.1% Tween20 (TBST or PBST) and blocked with 5% milk in TBST or PBST for 1 hr at room temperature. Protein bands were analyzed densitometrically using Scion Image software (Scion Corporation, Frederick, MD). The gray scale density readings were calibrated using a transmission step-wedge standard. The integrated optical density (IOD) of each band was calculated as the sum of the optical densities of all the pixels within the area of the band outlined. The IOD for the film background was subtracted from the IOD for each band. Each sample was measured in triplicate. RGS7 protein was normalized to actin protein and phosphoproteins were normalized to the corresponding total protein levels. Protein levels from treated cells were normalized to vehicle-treated cells for each western blot analysis.

Inositol phosphate (IP) accumulation assay:

The assay was performed as previously described (Singh, et al., 2007b). Briefly, cells were seeded in 24 well plates at a density of 40,000 cells/well. Cells were treated with vehicle or different concentrations of clozapine for 24 hr in serum-free DMEM media during the same 24-hr period; cells were labeled with 0.5 μCi [^{3}H] myoinositol/well. Following treatment and labeling, cells were washed 4 times with HBSS mix containing 20mM $LiCl_2$, and 20 mM HEPES, then incubated with 500 μL of same buffer at 37^{0}C for 15 min. Following the incubation, cells were challenged with 100 μM DOI for 30 min. Media was removed with 10mM ice-cold formic acid after the challenge treatment, and incubated on ice for 1 hr. AG1-X8 (Bio-Rad) resin columns were prepared as follows: columns were washed once with 3 ml of 3 M-ammonium formate/100 mm formic acid, twice with 5 ml of 10 mm formic acid/10 mm inositol. Once the columns were drained out completely, samples were loaded into the column and allowed to enter into the resin. Columns were then washed once with 5 ml of 10 mM formic acid/10 mM inositol, twice with 5 ml of 60 mM sodium formate/ 5 mM borax. After washing, samples were eluted with 5 ml of 1 M ammonium formate/ 100 mM formic acid into scintillation vials, 12 ml of scintillation cocktail was added into each vial, mixed thoroughly and counted in a scintillation counter.

PLC Assay:

Since preincubation with AG490 interferes with [^{3}H] myoinositol incorporation into A1A1v cells, we used an alternative, ex vivo, method to isolate membranes from control and treated cells and incubate the membrane fraction with [^{3}H] myoinositol. This method involves testing the enzymatic activity of PLC present in isolated membranes thereby avoiding any problems with incorporation of [^{3}H] myoinositol in presence of AG490. To harvest cells, culture plates were washed twice with Tris buffer (25 mM Hepes-Tris, 1mM EGTA, pH 7.4, containing protease inhibitor cocktail). Cells were harvested by scraping cells off the plates, and then cells were centrifuged at 20,000g for 20 min at 4°C. The pellet was resuspended in Tris buffer and stored at -80°C. Homogenates were thawed on the day of the PLC assay and homogenized by hand with five up-and-down strokes with a glass on glass homogenizer and then centrifuged at 20,000g for 20 min. After centrifugation, the pellet was resuspended in 50 mM Tris buffer and centrifuged at 20,000g for 10 min. The resultant pellet was washed 3X and resuspended in assay buffer (25 mM Tris pH 7.4, 3 mM EGTA, and 10 mM LiCl).

5-HT- and GTPγS-stimulated PLC activity in cell membranes were measures as described previously (Damjanoska, et al., 2003;Wolf and Schutz, 1997). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical). The membrane protein was diluted to an approximate concentration of 30 µg/100 µl with buffer containing 25

mM HEPES-Tris, 3 mM EGTA, 10 mM LiCl, 12 mM MgCl2, 1.44 mM sodium deoxycholate with 0.5 μM GTPγS (a nonhydrolyzable form of GTP), 100 nM free Ca2+, 1mM unlabeled phosphatidylinositol, and 100 μM [3H] phosphatidylinositol (PerkinElmer Life and Analytical Sciences). A concentration of 100 μM 5-HT or 1 μM of bradykinin was used to stimulate PLC activity. 5-HT-stimulated PLC activity is a selective measure of 5-HT_{2A} receptor function in A1A1v cells as previously demonstrated using selective antagonists (Shi, et al., 2007). Bradykinin was used to investigate the selectivity of olanzapine-induced desensitization of 5-HT_{2A} receptor signaling.

RNA Isolation and Reverse Transcription:

Total RNA was isolated using the RNeasy Mini Kit (Qiagen Sciences, Valencia, CA) according to the manufacturer's protocol. Total RNA was quantitated using a spectrophotometer and optical density (OD) 260/280 nm ratios were determined. Quality of the RNA was further accessed with a formaldehyde-agarose gel. First strand cDNA was synthesized using random hexamers and Superscript II Reverse Transcriptase from Invitrogen according to the manufacturer's protocol. Reactions were incubated at 25^0C for 2 min, 25^0C for 10 min, and 42^0C for 50 min and inactivated by heating at 70^0C for 15 min in an M J Mini, personal thermal cycler (BIO-RAD).

Real-Time PCR:

The GAPDH (sense 5'-tggagtctactggcgtcttcac-3'; antisense 5'-ggcatggactgtggtcatga-3') and RGS7 (sense 5'-gaagatgagttgcaccgacaga-3'; antisense 5'-ggtctttcagtgcctcatccat-3') primer sets were synthesized by IDT, Inc (Coralville, IA). PCR amplification was performed with 7500 Real-Time PCR System using SYBR green PCR master mix (Applied Biosystems, Foster city, CA). The PCR parameters used were a 10 min denaturation cycle at 95°C, 40 cycles of amplification at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Real-Time PCR was performed with 25 μL reaction mixture of cDNA, primers and SYBR green master mix.

RNA Data Analysis:

Comparative Ct (ΔΔCT) method was used for analysis of all real-time PCR data. ΔCT values were calculated by normalizing CT values of RGS7 to GAPDH from vehicle and antagonist-treated groups. The extent of the response is determined by $2^{mean(\Delta\Delta CT)}$, and the relative degree of response is calculated by $2^{-mean\ (\Delta\Delta CT)}$. Results are expressed as fold change in RGS7 mRNA levels for clozapine, MDL100907 or olanzapine-treated cells with respect to vehicle-treated cells. Data presented are from four independent experiments performed in triplicate.

Chromatin Immunoprecipitation Assay (ChIP):

The chromatin immunoprecip -itation (ChIP) assay was performed using a kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Following crosslinking, the DNA/protein complexes were sheared by sonication. One percent of sheared DNA/protein complex was kept and used as an input DNA sample. Anti-STAT3 rabbit polyclonal antibody (Sigma, Saint Louis, MO), or normal rabbit IgG (Millipore, Billerica, MA) was used for immunoprecipitation. Immunoprecipitated DNA/protein complexes were analyzed using polymerase chain reaction (PCR) with following primer sets flanking the five potential STAT3 binding sites (site 1-site 5):

F1 5'-GAAGTCAGGAGTCAGTCAAAGC-3',

R1 5'- ACTCCTTGGCTTCAACTAT GG-3'

F2 5'-AAGCTGGGTACGTTTCAGG-3', R2 5'-AATTTGGAGGCCTGGACC-3'

F3 5'-ATCCTTGGCACTGGACACC-3', R3 5'-GGGCTAAGATAATGGGAGG-3'

F4 5'-GATGGTTTGCCACTTGTGC-3', R4 5'- CTACTCTGCAGCCATCTGC-3'

F5 5'- ACATTCCAACAGGACCGG-3', R5 3'-ATCGGTCATGGCATCTCACC-3'

A previously identified STAT3 binding region from the hepcidin gene was used as a positive control (F 5'- GAGGGTGACACAACCCTGTT-3', R 5'- ACCGAGTGACA GTCGCTTTT-3') (Wrighting and Andrews, 2006). Two microliter of precipitated DNA was amplified using Taq polymerase (New England, Biolabs, Ipswich, MA). The conditions for PCR amplification were as

follows: 40 cycles of 94^0C for 15 s, 55^0C for 15 s, 72^0C for 45 s, and the amplimers were resolved on 1% agarose gels containing ethidium bromide.

Statistics:

All statistical analyses were performed using GB-STAT School Pak (Dynamic Microsystems, Silver Spring, MD). Data are expressed as means ± SEM. For Western blots and IP accumulation assay, data were analyzed using a using a one-way analysis of variance, followed by a Newman–Keuls' post hoc analysis. RT-PCR and PLC activity assay was analyzed using a two-way analysis of variance, followed by a Newman–Keuls' post hoc analysis.

Results

IP3 accumulation assay:

Agonist-stimulated IP3 accumulation can be used to monitor desensitization of 5-HT_{2A} receptor signaling (Singh, et al., 2007). Treatment with different concentrations of clozapine for 24 h significantly decreased ($F_{(4,14)}$ = 104.43, p < 0.0001) DOI (10^{-4} M) stimulated IP3 accumulation in a dose dependent manner compared to vehicle (DMSO) treated cells (figure 11). A post-hoc analysis revealed a decrease in IP3 accumulation by 39% with 5 μM ($p < 0.01$), 53% with 20μM ($p < 0.01$), 64% with 30μM ($p < 0.01$), and 80% with 40 μM ($p<0.01$) treatment. This decrease suggests a

desensitization of 5-HT_{2A}-mediated receptor signaling in A1A1v cells by clozapine.

Clozapine and MDL100907 treatment increase phosphorylation of JAK2:

We have shown that the 5-HT_{2A} receptor inverse agonist, olanzapine causes phosphorylation of JAK2 kinase (Singh, et al., 2007). In order to investigate whether the effect is specific to olanzapine or is a general effect of atypical antipsychotics and more specifically 5-HT_{2A} receptor antagonists, we treated A1A1v cells for 24h with either clozapine, MDL100907 or drug vehicles. Membrane fractions prepared from vehicle, clozapine (20μM), and MDL100907 (1μM) treated cells were analyzed by western blot with an anti-phospho-JAK2 antibody, then stripped and reprobed with an anti-JAK2 antibody (figure 12). Tyrosine phosphorylation of JAK2 was significantly increased ($F_{(2,8)} = 39.57$, $p < 0.001$). A post-hoc analysis revealed that pJAK levels were increased to 183 ±13 % of the control levels with MDL100907 and 196±18% of the control levels with clozapine treated cells, whereas total JAK2 protein levels did not show any appreciable change.

Clozapine and MDL100907 increase RGS7 protein levels:

Next, we wanted to determine whether this increase in JAK2 phosphorylation with both clozapine and MDL100907 is also accompanied by increases in RGS7 protein levels as previously observed with olanzapine

(Singh, et al., 2007). In order to monitor the changes in RGS7 protein levels, membrane fractions of vehicle, clozapine, or MDL100907-treated cells were analyzed by western blot with anti-RGS7 antibody (figure 13). We found that RGS7 protein levels were significantly increased ($F_{(2,8)}$ = 95.99, p < 0.001) by drug treatments. A post-hoc analysis revealed that RGS7 protein levels were increased to 176 ±16 % of the control levels with MDL100907 and 194±11 in clozapine treated cells.

JAK2 inhibitor partially reversed the olanzapine-induced desensitization of 5-HT_{2A} receptor signaling:

We have previously shown that olanzapine-induced activation of the JAK2-STAT3 pathway is necessary for the increase in RGS7 protein levels; next we wanted to determine if activation of the JAK2-STAT3 pathway is necessary for olanzapine-induced desensitization of 5-HT_{2A} receptor signaling. However, AG490 interfered with the measurements of IP3 accumulation in vivo. Therefore, we decided to use an alternative ex vivo method in which we isolated membranes from AG490 pretreated, control and olanzapine-treated cells and then incubated them with [^{3}H] myoinositol. This method involves measuring the enzymatic activity of PLC present in isolated membranes thereby avoiding any interference of preincubation of AG490 on incorporated [^{3}H] myoinositol. Olanzapine-induced changes in PLC activity were monitored in response to stimulation with 5-HT, bradykinin, or GTPγS. 5-

HT stimulation measures the ability of 5-HT_{2A} receptors to stimulate PLC activity via Gαq/11 activation where as GTPγS directly binds to $G_{\alpha q/11}$ to activate PLC. Bradykinin was used to investigate the specificity of olanzapine-induced desensitization response by examining the effects on another $G_{q/11}$ linked receptor system. Olanzapine treatment significantly decreased (by 55%, $p<0.01$). PLC activity stimulated with serotonin. AG490 alone had no effect on PLC activity (figure 14A). However, pretreatment with AG490 significantly attenuated the olanzapine-induced decreases in PLC activity ($p<0.05$) suggesting that the JAK-STAT pathway is necessary for the full olanzapine-induced desensitization of 5-HT_{2A} receptor signaling. Two-way ANOVA indicates a main effect of olanzapine on PLC activity ($F_{(1,19)} = 41.18$, $p < 0.001$), a main effect of AG490 ($F_{(1,19)} = 4.23$, $p < 0.05$), but no significant interaction was observed between olanzapine and AG490 ($F_{(1,19)} = 2.92$, $p < 0.391$). In addition, olanzapine or AG490 treatment had no effect on GTPγS-stimulated PLC activity (figure 14B). When olanzapine-treated cells were stimulated with bradykinin (figure 14C); olanzapine treatment had no effect on bradykinin-stimulated PLC activity confirming that olanzapine treatment specifically desensitizes 5-HT_{2A} receptor signaling.

Olanzapine, Clozapine and MDL100907 increase mRNA levels of RGS7 protein:

To investigate whether the increase in RGS7 protein levels is associated with an increase in RGS7 mRNA levels, cells were treated in a similar fashion as in previous experiments for 24h with vehicle, olanzapine, clozapine, or MDL100907. Total RNA was isolated from each sample and reverse transcribed with random hexamers to make cDNA. Using that cDNA, a real-time PCR analysis was performed with specific primers for RGS7 and GAPDH. The vehicle and drug-treated RGS7 mRNA levels were normalized to GAPDH. We found a statistically significant ($F_{(1,15)} = 43.43$; $p < 0.001$) increase in RGS7 mRNA levels. A post-hoc analysis revealed that RGS7 mRNA levels were increased to 153±11% with olanzapine (figure 15A), 175±14 % with clozapine, and 144±17 % with MDL100907 treatment (figure 15B) over their respective vehicle-treated cells.

STAT3 binds to the putative RGS7 promoter region:

To determine if STAT3 binds to potential STAT3 consensus site in cells, we used a ChIP approach. A bioinformatic analysis of the 10 kb promoter region of rat RGS7 identified five potential STAT3 binding sites based on the consensus sequence-$TTCN_{2-4}GAA$. Specific primers were designed that flank each of these consensus sites and were named site number one through five (figure 6). A1A1v cells were treated with vehicle (20% acetic

acid) or olanzapine (300nM) for 24h as previously described. Immunoprecipitates isolated from either vehicle or olanzapine-treated cells were used to perform ChIP analysis. Of the five potential STAT3 binding sites identified, only site 2 tested positive in this analysis (Figure 6). Moreover, treatment with olanzapine produced increased STAT3 binding at this site. Site 2 begins 2.34kb upstream of the RGS7 transcription start site. The primers specific for hepcidin, used as a positive control, also tested positive using STAT3 immunoprecipitates. In contrast, when the beads alone or pre-immune IgG was used to produce chromatin no amplimer was detected.

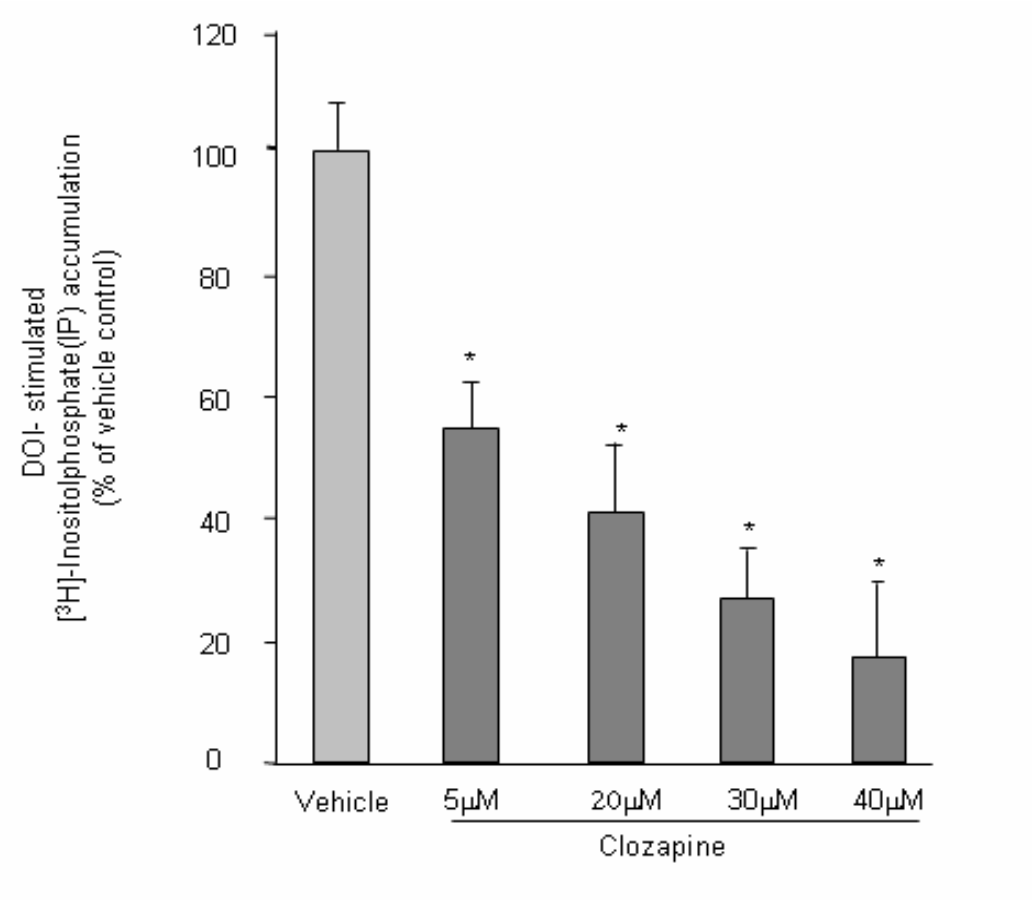

Figure 11. Clozapine decreases DOI-stimulated IP accumulation. A1A1v cells were treated with either vehicle (DMSO) or with various concentrations of clozapine for 24 h, and incubated with [3H]-myoinositol for same 24 h period. Both vehicle- and clozapine-treated cells were stimulated with 10^{-4} M DOI. Bar graph represents DOI-stimulated IP accumulation normalized to DOI-stimulated IP accumulation in vehicle-treated cells from four independent experiments. IP accumulation was significantly decreased in clozapine-treated cells compared to vehicle-treated cells. * indicates significantly different from vehicle-treated cells at $p < 0.01$.

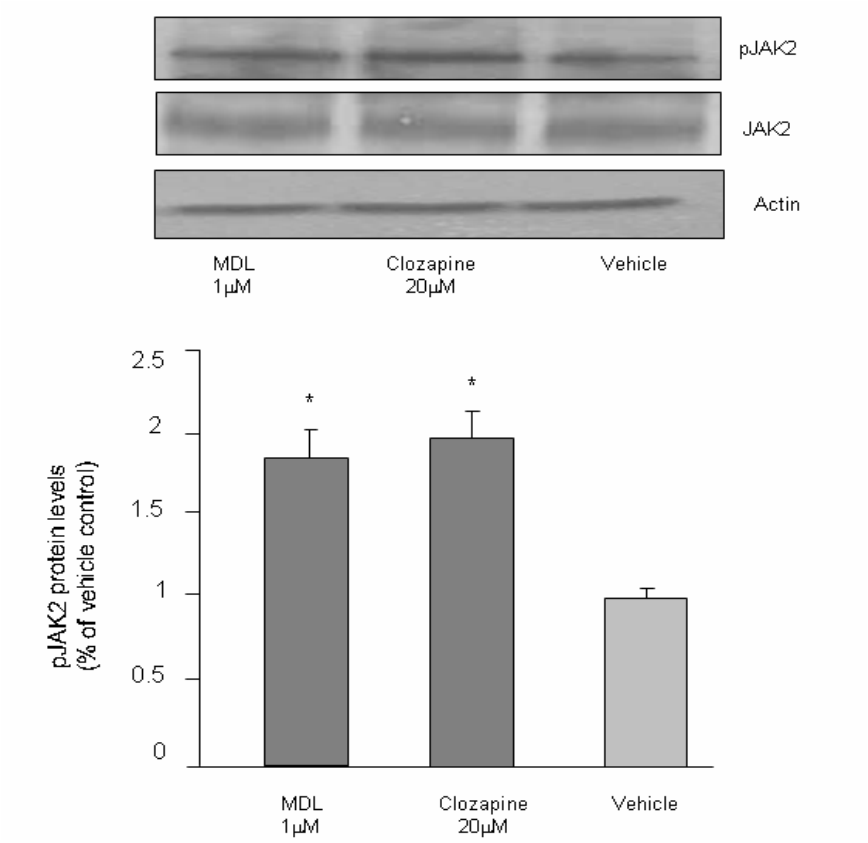

Figure 12. Clozapine and MDL100907 stimulate JAK2 phosphorylation. A1A1v cells were treated either with vehicle (DMSO) or 20 μM clozapine or 1μM MDL100907 for 24 h. Membrane fractions of cells were analyzed by western blot with anti-phosphoJAK2 antibody, stripped and reprobed with anti-JAK2 and anti-actin antibodies. Bar graph represents quantification of phosphoJAK2 protein levels divided by JAK2 protein levels from five independent experiments. Phosphorylation of JAK2 was significantly increased ($p<0.05$) with both clozapine and MDL100907 treatment compared with vehicle treated cells. * indicates significantly different from vehicle-treated cells at $p < 0.05$.

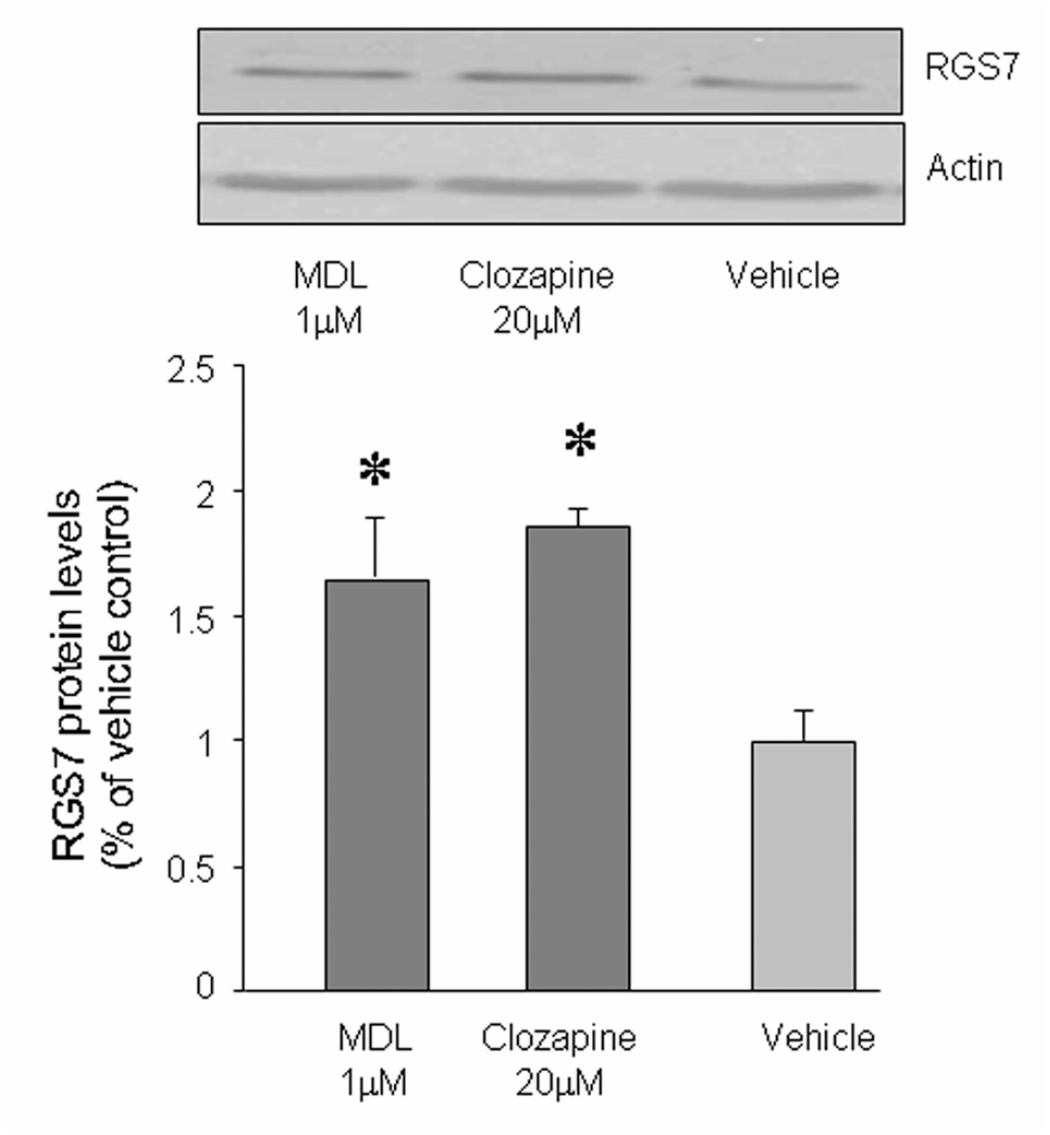

Figure 13. Clozapine and MDL100907 treatment increases RGS7 levels. A1A1v cells were treated either with vehicle (DMSO) or 20 μM clozapine or 1μM MDL100907 for 24 h. Membrane fractions of cells were analyzed by western blot with an anti-RGS7 antibody, stripped and reprobed with an anti-actin antibody as a loading control. Bar graph represents quantification of RGS7 protein levels divided by actin protein levels from four independent

experiments. RGS7 protein levels were significantly increased ($p<0.05$) with clozapine and MDL100907 compared to vehicle-treated cells. * indicates significantly different from vehicle-treated cells at $p < 0.05$.

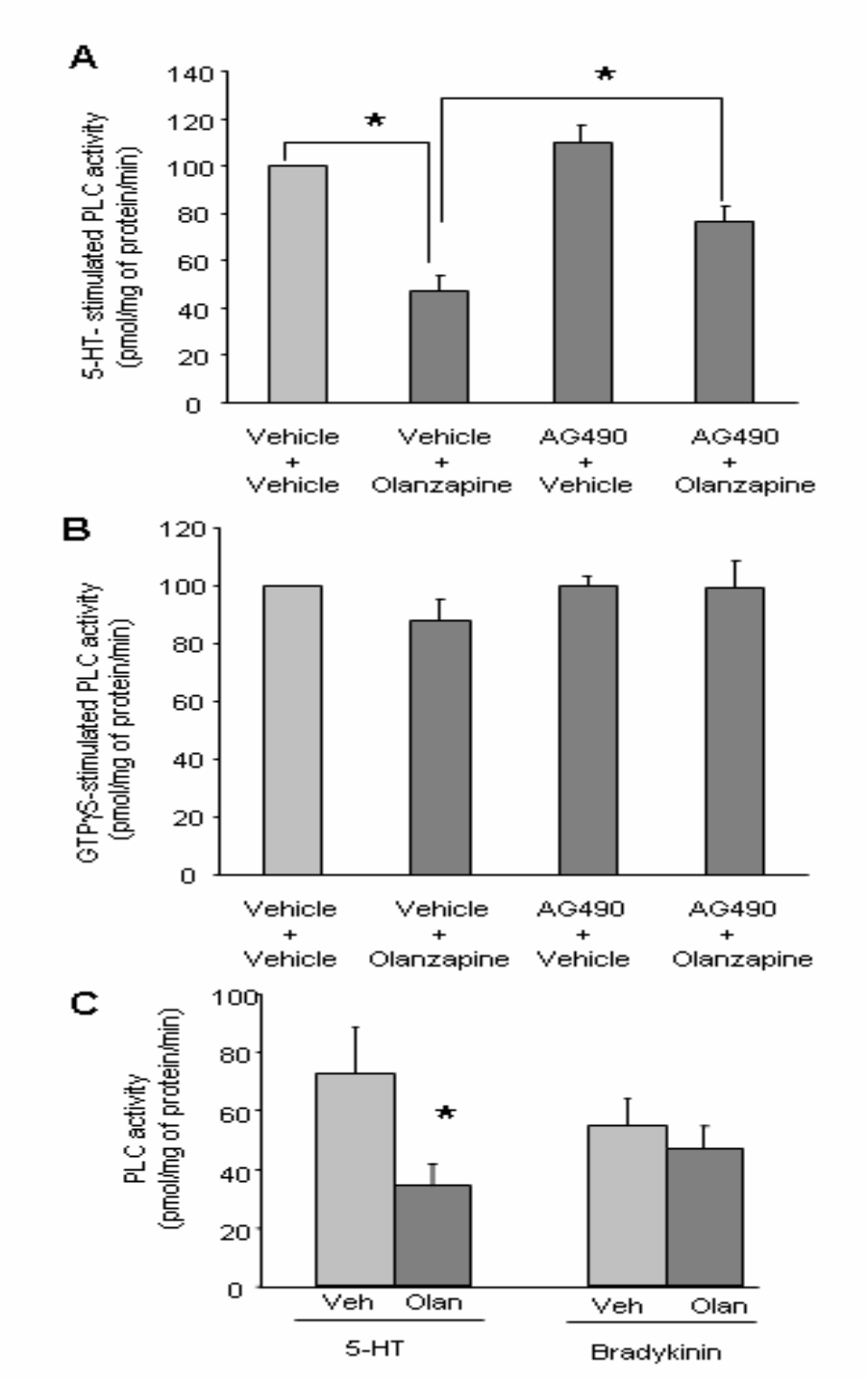

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Figure 14. A JAK inhibitor partly attenuated the olanzapine-induced decrease in PLC activity. A1A1v cells were pretreated for 1h with the 30μM AG490 prior to treating with either vehicle (20% acetic acid) or 300nM olanzapine for 24 h. (A) 5-HT-stimulated PLC activity was significantly ($p<0.01$) reduced by olanzapine treatment compared to vehicle-treated control (*indicates significantly different from vehicle-treated control at $p < 0.01$). AG490 alone did not have any effect on PLC activity. However, in cells pretreated with AG490, the olanzapine-induced decrease in PLC activity was significantly attenuated ($p<0.05$) (B) GTPγS-stimulated-PLC activity was not altered either by the olanzapine or AG490 treatments. (C). Olanzapine treatment had no effect on Bradykinin-stimulated PLC activity, whereas 5-HT-stimulated PLC activity was significantly reduced suggesting olanzapine treatment selectively affect 5-HT_{2A} receptor mediated-PLC activity.

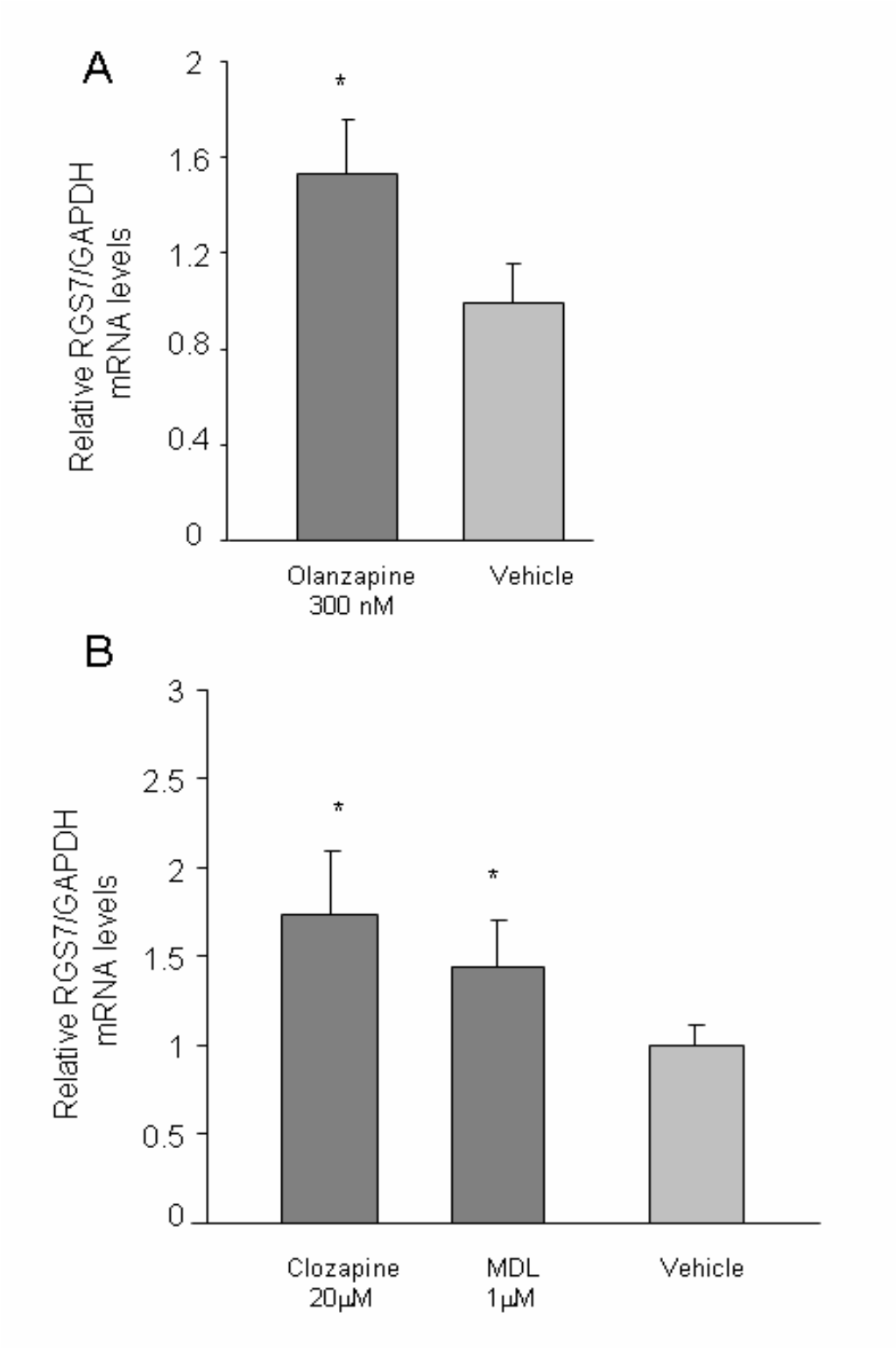

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Figure 15. Olanzapine, clozapine and MDL100907 increase RGS7 mRNA levels. A1A1v cells were treated either with vehicle (20% acetic acid), 300nM olanzapine, vehicle (DMSO), 20 μM clozapine or 1μM MDL100907 for 24 h. Total RNA was isolated from vehicle-treated control or drug-treated cells, and equal amounts of cDNA were reverse-transcribed. Bar graph represents quantification of RGS7 mRNA levels normalized to GAPDH levels from five independent experiments and represents the fold change over control. RGS7 mRNA levels were significantly increased in olanzapine, clozapine and MDL100907-treated compared to vehicle-treated cells ($p<0.05$). * indicates significantly different from vehicle-treated cells at $p<0.05$.

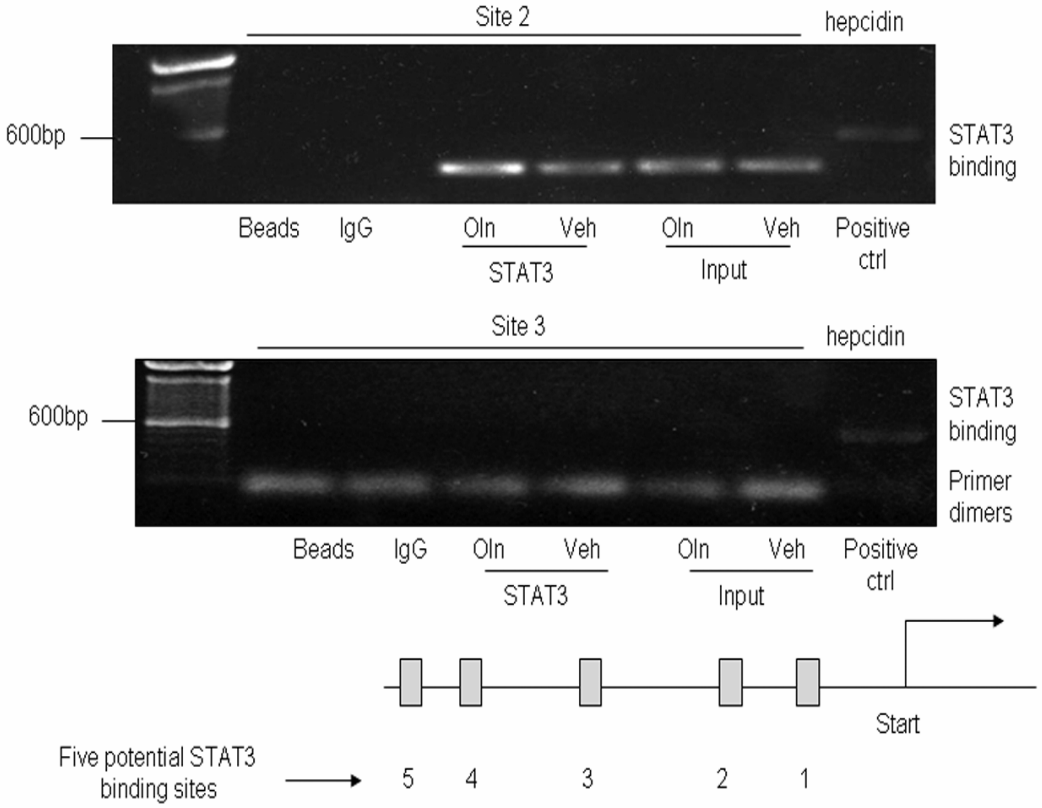

Figure 16. STAT3 binds to the putative RGS7 promoter region at site 2. ChIP assays were performed as described in “Materials and Methods” with an anti-STAT3 antibody, non-immune IgG, or with beads alone. DNA samples were amplified using primer pairs that flanked all five potential STAT3-binding sites (site1 – site 5). Primers that amplify the STAT3 binding site previously identified in the hepcidin gene were used as a positive control. Non-precipitated genomic DNA (input) was used as a positive control. Amplification was not observed in lanes loaded with samples immunoprecipitated with beads alone, i.e., without the STAT3 antibody, and

non-specific IgG in place of the STAT3 antibody confirming specificity of the reaction with the STAT3 antibody. For brevity, only site 2 and site 3 results are shown.

Discussion

Atypical antipsychotics have been previously reported to act as inverse agonist and to induce desensitization of 5-HT_{2A} receptor signaling (Egan, et al., 1998;Egan, et al., 2000;Herrick-Davis, et al., 1998;Rauser, et al., 2001). Internalization and down-regulation have been proposed as mechanisms of desensitization, (Roth, et al., 1995;Willins, et al., 1998) however, subsequent studies provided evidence supporting both internalization- and down-regulation- dependent and independent desensitization of 5-HT_{2A} receptor by atypical antipsychotics (Kuoppamaki, et al., 1995;Hanley and Hensler, 2002). It is likely that internalization and down-regulation can contribute to the desensitization process but whether they are necessary and sufficient for the full desensitization process is not known. In addition, studies were inconclusive regarding the role for transcriptional regulation of 5-HT_{2A} receptor down-regulation with antipsychotics (Gray and Roth, 2001). Both, a decrease in receptor mRNA in hippocampus, brain stem, and midbrain and no change in mRNA levels are reported previously with atypical antipsychotics (Burnet, et al., 1996;Doat-Meyerhoefer, et al., 2005). Thus, transcriptional regulation of 5-HT_{2A} receptor by atypical antipsychotics does not appear to be responsible for down-regulation leading to the desensitization of 5-HT_{2A} receptor signaling.

Consistent with previous reports that atypical antipsychotics induce desensitization of 5-HT_{2A} receptor signaling, (Gray and Roth, 2001) we find

that olanzapine and clozapine induce desensitization of 5-HT_{2A} receptor signaling in A1A1v cells. Olanzapine, clozapine, and MDL100907 increase RGS7 mRNA and protein levels as well as the activation of the JAK-STAT pathway. We previously found that the increase in RGS7 protein expression in response to olanzapine treatment is dependent of JAK-STAT signaling (Singh, et al., 2007). We now further report that the desensitization response on the 5-HT_{2A} receptor signaling, induced by these atypical antipsychotics is dependent on JAK-STAT signaling. Olanzapine-induced decreases in PLC activity, monitored as an index of 5-HT_{2A} receptor responsiveness (i.e., desensitization), were significantly attenuated by pretreatment with a JAK2 kinase inhibitor. These data suggest that activation of the JAK-STAT pathway is necessary for atypical antipsychotics-induced 5-HT_{2A} receptor-mediated PLC activity.

In addition to 5-HT_{2A} receptors, atypical antipsychotics also have high affinity for other GPCRs for example, other 5-HT receptors (e.g., 5-HT_{1A}, 5-HT_{2C}, 5-HT_6, and 5-HT_7) (Meltzer, et al., 1989;Roth, et al., 1992;Meltzer, 1999;Seeger, et al., 1995), the dopamine D_4 receptor (Van Tol, et al., 1991;Roth, et al., 1995), all five muscarinic receptors (m1-m5) (Peroutka and Synder, 1980;Zeng, et al., 1997), and several adrenergic and histamine receptors (Peroutka and Synder, 1980). MDL100907 initially characterized as a selective antagonist of 5-HT_{2A} receptor, has been used to delineate antipsychotic responses mediated specifically by 5-HT_{2A} receptor (Olijslagers,

et al., 2005;Wolff and Leander, 2000;Bhagwagar, et al., 2006) and desensitizes 5-HT_{2A} receptor signaling (Rauser, et al., 2001;Shi, et al., 2007). In this study, we report that MDL100907 stimulated activation of the JAK-STAT pathway and increased RGS7 protein and mRNA levels suggesting that antagonism of 5-HT_{2A} receptors is sufficient to induce these changes caused by atypical antipsychotics.

RGS proteins reduce G-protein-mediated signaling by acting as guanosine triphosphatase (GTPase)-accelerating proteins (GAPs) for G_{α} subunits (Hollinger and Hepler, 2002;Dohlman and Thorner, 1997). Expression of RGS7 protein in rat frontal cortex is well documented (Zhang and Simonds, 2000c;Krumins, et al., 2004) and decreased 5-HT_{2A} receptor signaling via direct interaction of RGS7 protein with $G_{\alpha q}$ has been characterized in different systems (DiBello, et al., 1998;Ghavami, et al., 2004). Furthermore, an increase in RGS7 protein following both olanzapine and clozapine treatment would increase the termination rate of 5-HT_{2A} receptor- $G_{\alpha q/11}$ protein signaling by more rapidly hydrolyzing GTP, and could thereby produce or contribute to the desensitization response. When GTPγS, a non-hydrolysable GTP analog is used to activate G proteins, PLC activity is not affected by RGS proteins, since RGS proteins are not able to hydrolyze GTPγS. Therefore, the differential effects of olanzapine on receptor versus G-protein activation of PLC activity are consistent with an increase in RGS7 protein as an underlying mechanism for olanzapine-induced desensitization. The

increased levels of RGS7 could reduce 5-HT-stimulated PLC activity as we found in olanzapine treated cell membranes; however, GTPγS stimulated-PLC activity was not altered by olanzapine consistent with increase RGS proteins underlying the change in response.

Numerous studies have reported a significant decrease in RGS4 expression in the prefrontal cortex of schizophrenic subjects (Mirnics, et al., 2001;Erdely, et al., 2006;Bowden, et al., 2007;Gu, et al., 2007). Expression of RGS4 and RGS7 have been previously noted to be independent (Krumins, et al., 2004). Like RGS7 proteins, RGS4 also regulates 5-HT_{2A} receptor signaling. Atypical antipsychotic-induced increases in RGS7 levels observed in our studies might restore the 5-HT_{2A} receptor signaling duration to physiological levels by substituting for the diminished RGS4 protein.

Atypical antipsychotics could increase RGS7 levels by either increased stability of RGS7 protein or by increased transcription of RGS7 mRNA. RGS7 binding to $G_{\beta 5}$ is reported to increase the stability of each protein (Chen, et al., 2003). In addition, RGS7 phosphorylation and subsequent binding to 14-3-3 sequesters RGS7 in the cytoplasm (Burchett, 2003). Therefore, an increase in phosphorylation of RGS7 or increased expression of 14-3-3 or $G_{\beta 5}$ could increase the levels of RGS7 in the cytoplasm. Our real-time PCR data suggest that the increase in RGS7 levels by olanzapine, clozapine and MDL100907 could be directly mediated by an increase RGS7 mRNA via activation of the JAK-STAT pathway. STAT3 regulates a variety of biological

processes, functioning at both transcriptional and non-transcriptional levels to influence cell growth, survival and metabolism (Inghirami, et al., 2005;Leeman, et al., 2006). From a genomic sequence analysis of rat RGS7, we have identified multiple sets of the STAT3 consensus binding element, $TTCN_{2-4}GAA$, (Decker, et al., 1997;Ehret, et al., 2001), suggesting that STAT3 could be a possible transcription factor for the RGS7 promoter. Using a ChIP analysis, we found one of the STAT3 consensus binding elements located at 2.34kb upstream of transcription start site strongly binds with STAT3 in response to olanzapine treatment. STAT3 binding to the RGS7 gene along with an increase in mRNA levels of RGS7 suggests the possibility that STAT3 is a transcription factor for RGS7. The promoter region of RGS7 is not yet identified, however, it is usually present upstream of the transcription start site consistent with our identified STAT3 binding site 2.34kb upstream from the transcription initiation site. Taken together, these results are consistent with our hypothesis that activation of JAK-STAT pathway by atypical antipsychotics and the subsequent increase in RGS7 expression is an underlying mechanism for desensitization of 5-HT_{2A} receptor signaling.

In our previous studies (Singh, et al., 2007;Muma, et al., 2007) we have reported olanzapine-induced activation of the JAK-STAT pathway. In this study we demonstrate that activation of the JAK-STAT pathway is necessary for full desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics in A1A1v cells. While the precise mechanism of JAK-STAT activation by

atypical antipsychotics is not determined, it has been reported that 5-HT_{2A} receptor associates in a complex with JAK2 and STAT3 (Guillet-Deniau, et al., 1997). Other studies have reported that 5-HT activates JAK2, JAK1, and STAT1 via the 5-HT_{2A} receptors (Banes, et al., 2005). Furthermore, atypical antipsychotics have also been reported to activate other signaling cascades for example, activation of ERK1/2 pathways in the rat frontal cortex (Fumagalli, et al., 2006), Akt/PKB and P38 pathways in PC12 cells (Lu, et al., 2004), and GSK3α/β in the rat frontal cortex (Kang, et al., 2004;Roh, et al., 2007). Although, atypical antipsychotics have been extensively characterized as an inverse agonist/antagonist of 5-HT_{2A} receptor, activation of JAK-STAT in our studies clearly indicates that besides being an antagonist, atypical antipsychotics are agonist for the JAK-STAT pathway. Our studies demonstrate that agonist activity as demonstrated by activation of JAK-STAT pathway and antagonist effects at the PLC enzyme occur simultaneously. Previous studies have demonstrated selective agonism, where one agonist stimulates one pathway preferentially over another (Kenakin, 2007). Our studies extend the diversity of signaling by a single receptor suggesting that a ligand like olanzapine or clozapine can be an agonist for one 5-HT_{2A} receptor mediated pathway, JAK-STAT, and simultaneously an antagonist at the $G_{q/11}$-PLC pathway.

Overall, our data suggest that desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics requires activation of the JAK-STAT

pathway. In addition, activation of the JAK-STAT pathway and increases in RGS7 expression by transcriptional activity of STAT3 is likely to contribute to the full desensitization response of 5-HT_{2A} receptors signaling. However, further studies are needed to confirm the transcriptional activity of STAT3 on putative promoter site of RGS7.

CHAPTER V

JAK-STAT PATHWAY IN OLANZAPINE-INDUCED DESENSITIZATION OF 5-HT_{2A} RECEPTOR SIGNALING IN RAT BRAIN

Abstract

We previously demonstrated that chronic treatment with olanzapine causes desensitization of 5-HT_{2A} receptor signaling in the rat frontal cortex and this desensitization response is associated with increases in RGS7 protein levels and activation of STAT3 (Muma, et al., 2007). We now report that desensitization of 5-HT_{2A} receptor signaling induced by olanzapine is dependent on activation of the JAK-STAT pathway. Pretreatment with a JAK2 inhibitor significantly attenuated 5-HT_{2A} stimulated-PLC activity. In the current study, we found that olanzapine treatment increased RGS7 protein levels (211±19% of control levels), RGS7 mRNA levels (188±13%), and activation of JAK2 (198±14% of control levels) in rat frontal cortex. Furthermore, inhibition of JAK activation also prevented the olanzapine-induced increases in RGS7 mRNA and protein levels. We verified that treatment with AG490 reduced phosphorylation of JAK2. The functional status of the 5-HT_{2A} receptor pathway in the hypothalamic paraventricular nucleus (PVN) was examined using 5-HT_{2A} receptor-stimulated increases in plasma hormone levels. Plasma oxytocin, adrenocorticotrophic hormone (ACTH) and corticosterone measurements showed that olanzapine injections for 7 days caused

desensitization of 5-HT_{2A} receptor signaling (oxytocin response decreased 81.63±4.37%; ACTH response decreased 38.68±12.43%; corticosterone response decreased 54 ± 6.41%). Surprisingly, oxytocin and corticosterone levels were also decreased in a dose-dependent manner by the JAK2 inhibitor whereas ACTH levels were not altered. Taken together, these results suggest that the olanzapine-induced increase in RGS7 expression is mediated by activation of JAK-STAT and is necessary for olanzapine-induced desensitization of 5-HT_{2A} receptor signaling in the frontal cortex. Although, decreases in plasma hormone levels confirmed olanzapine-induced desensitization of 5-HT_{2A} receptor signaling in the hypothalamus, the precise role of the JAK-STAT pathway in 5-HT_{2A}-stimulated hormonal release requires further investigation.

Introduction

Evidence implicates involvement of serotonin 5-HT_{2A} receptors in a number of psychiatric disorders, including schizophrenia, depression, and anxiety(Nichols, et al., 1994;Naughton, et al., 2000). Furthermore, atypical antipsychotics are reported to bind with high affinity at 5-HT_{2A} receptors as determined in cell culture (Meltzer, et al., 1989) and in vivo (Zhang and Bymaster, 1999). Although, atypical antipsychotics bind to diverse population of receptors (Meltzer, et al., 1989;Altar, et al., 1986;Zhang and Bymaster, 1999;Roth, et al., 2004), therapeutic benefits associated with atypical

antipsychotics are attributed, in part, to their ability to antagonize (i.e. desensitize) 5-HT_{2A} receptor signaling (Goyer, et al., 1996). In addition, further evidence for the involvement of 5-HT_{2A} receptors in the mechanisms of action of atypical antipsychotics came from reports of polymorphisms in the promoter and coding regions of the 5-HT_{2A} receptor gene. Schizophrenics with this polymorphism were reported to respond poorly to clozapine treatment suggesting that genetic variation at 5-HT_{2A} receptors may influence clozapine response and strengthens the candidacy of these receptors as important therapeutic targets (Yu, et al., 2001;Arranz, et al., 1996;Masellis, et al., 1998).

Several studies have reported the desensitization and down-regulation of central 5-HT_{2A} receptor signaling by antagonist in the brain (Blackshear and Sanders-Bush, 1982;Gandolfi, et al., 1985;Hensler and Truett, 1998),however, molecular mechanisms underlying these changes are not understood. In addition, atypical antipsychotics are reported to reduce levels of ACTH and cortisol in schizophrenic patients (Cohrs, et al., 2006;Scheepers, et al., 2001;Hatzimanolis, et al., 1998;Meltzer, et al., 1989;Hatzimanolis, et al., 1998). Although, monoaminergic mechanisms including serotonin and dopamine are known to play an important role in the regulation of ACTH and cortisol secretion (Wilcox, et al., 1975;Fuller and Snoddy, 1984;Tuomisto and Mannisto, 1985;Lefebvre, et al., 1998), the attenuation of cortisol secretion, after subchronic administration of olanzapine

and clozapine to schizophrenic patients, has been attributed to 5-HT receptor blockade(Scheepers, et al., 2001;Meltzer, et al., 1989;Hatzimanolis, et al., 1998;Hatzimanolis, et al., 1998). Furthermore, 5-HT_{2A} receptors in the hypothalamic paraventricular nucleus have been shown to mediate the neuroendocrine responses to a peripheral injection of DOI, intraparaventricular and peripheral injections of the selective 5-HT_{2A} receptor antagonist MDL 100,907 dose-dependently inhibit the DOI-induced increases in hormone secretions (Zhang, et al., 2002). These results suggest that alterations in hormone secretions by atypical antipsychotics may be mediated by 5-HT_{2A} receptors and that plasma hormone levels can provide an index of the function of 5-HT_{2A} receptor signaling in the hypothalamic paraventricular nucleus.

5-HT_{2A} receptors are coupled through $G_{q/11}$ proteins to phospholipase C (PLC) (Hide, et al., 1989;Roth, et al., 1998). Upon activation of PLC, hydrolysis of phosphatidylinositol 4,5-bisphosphate generates diacylglycerol and inositol 1,4,5-trisphosphate (Berridge, 1986). $G_{\alpha q/11}$ proteins stimulate PLC activity until the bound GTP is hydrolyzed to GDP. The intrinsic GTPase activity of $G_{\alpha q/11}$ proteins is enhanced by regulators of G protein signaling proteins type 4 and 7 (RGS4 and RGS7)(Hepler, et al., 1997;Xu, et al., 1999).

Activation of various GPCRs like angiotensin II, bradykinin, thrombin, and 5-HT_{2A} have been reported to induce activation of the JAK-STAT pathway under different cellular milieu and in different cell types (Ju, et al.,

2000;Marrero, et al., 1995;Guillet-Deniau, et al., 1997). Although, the exact mechanism by which 5-HT_{2A} receptors activate the JAK-STAT pathway is not known, 5-HT_{2A} receptors associate with JAK and STAT and rapid activation of JAK and STAT via phosphorylation in response to serotonin was previously reported (Guillet-Deniau, et al., 1997). In addition, chronic exposure to cocaine, an inhibitor of serotonin reuptake, was reported to activate JAK2 in the rat ventral tegmental area (VTA)(Berhow, et al., 1996). Previously, we have reported that daily treatment with olanzapine for 7 days causes desensitization of 5-HT_{2A} receptor signaling accompanied by activation of the JAK-STAT pathway and increases in RGS7 protein levels (Muma, et al., 2007). The JAK-STAT pathway regulates expression of a number of genes including c-Fos, c-Jun and c-Myc (Burysek, et al., 2002;Cattaneo, et al., 1999), transcription factors which can then stimulate expression of select genes. Consistent with these reports, we have also shown that olanzapine treatment in cells in culture causes JAK-STAT dependent desensitization and increases in membrane-associated RGS7 protein levels (Singh, et al., 2007). The increased membrane-associated RGS7 protein can then increase hydrolysis of activated $G_{\alpha q/11}$ and could contribute to the desensitization of 5-HT_{2A} receptor signaling. However, whether olanzapine-induced activation of JAK-STAT pathway has any direct impact on desensitization of 5-HT_{2A} receptor signaling in vivo is currently unknown.

Based on our previous studies, we hypothesized that olanzapine induces activation of the JAK-STAT pathway, and subsequent activation and translocation of STAT3 to the nucleus increases RGS7 transcription and mRNA thereby increasing RGS7 protein levels in the membrane. The increased membrane-associated RGS7 protein can then increase hydrolysis of activated $G_{\alpha q/11}$ and result in desensitization of 5-HT_{2A} receptor signaling. We began to test this hypothesis by investigating the impact of blocking the JAK-STAT pathway with a specific JAK2 inhibitor, AG490, on olanzapine-induced desensitization of 5-HT_{2A} receptor signaling in the frontal cortex and hormone release in the hypothalamus. Furthermore, we also monitored the effect of AG490 on olanzapine-induced increases in RGS7 protein levels and the impact of activated STAT3 on levels of RGS7 mRNA and protein in the frontal cortex.

Materials and Methods

Animals:

Male Sprague-Dawley rats (250–275 g; Harlan, Indianapolis, IN) were housed two per cage in an environment controlled for temperature, humidity, and lighting (7:00 AM–7:00 PM). Food and water was provided ad libitum. Eight rats were used per experimental group. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use

of Laboratory Animals as approved by the Loyola University Institutional Animal Care and Use Committee.

Drugs:

Olanzapine and AG490 were purchased from Torrent Research Chemicals Inc., ON, Canada. Olanzapine was dissolved in 20% glacial acetic acid and the pH was adjusted to 6.0 with 10M NaOH as described previously (Singh et al., 2007). Olanzapine was injected at a dose of 10 mg/kg i.p. AG490 was dissolved in 50% DMSO and injected at 2 mg/kg and 10 mg/kg s.c. DOI was purchased from Sigma/RBI (Natick, MA) and dissolved in 0.9% saline and injected at a dose of 1 mg/kg i.p. for the challenge injections.

Experimental procedures:

Rats were randomly assigned to the various experimental groups with cage mates being placed within the same experimental groups. The body weight of each rat was recorded every alternate day. They were kept in a quiet environment to minimize stress and prevent plasma hormones from exceeding basal levels. Rats were first injected with 50% DMSO or one dose of AG490 (2 mg/kg or 10 mg/kg s.c.). One hr later these rats were injected with either the vehicle 20% glacial acetic acid or 10 mg/kg olanzapine. These injections were repeated for 7 days. Twenty-four hr after the last injection of AG490 or olanzapine, eight rats from each group received either a challenge injection of (-)-DOI (1 mg/kg s.c.) or a 0.9% saline (1 ml/kg s.c.) 30 min before

sacrifice. The trunk blood was collected in centrifuge tubes containing 0.5 ml of 0.3 M EDTA (pH 7.4) solution. The plasma samples for radioimmunoassays were stored at -80°C. Whole brains were removed, frozen on dry ice, and stored at -80°C for biochemical and molecular analyses.

PLC activity assay:

The membrane fraction prepared from frontal cortex of animals challenged with saline was used for the measurement of PLC activity. PLC activity was measured by the amount of inositol 1,4,5 trisphosphate produced by PLC, as described previously (Damjanoska, et al., 2004;Wolf and Schutz, 1997a). Briefly, 30 µg of membrane protein from frontal cortex was diluted into 100 µl of total volume with a buffer containing 25 mM Hepes-Tris, 3 mM EGTA, 10 mM LiCl, 12 mM $MgCl_2$, 1.44 mM sodium deoxycholate with 1 µM GTPγS, 300 nM free Ca^{2+}, 1 µM 5-HT, and 1 mM unlabeled phosphatidyl inositol. The reaction tubes were kept on ice until the incubation period (20 min at 37°C) was started with the addition of 100 µM [^{3}H] phosphatidyl inositol. The reaction was stopped by addition of 0.9 ml of $CHCl_2$/MeOH (1:2) and 0.3 ml of chloroform. The tubes were shaken vigorously for 90 seconds and centrifuged at room temperature for 90 seconds at 13,600Xg. Then, 0.3 ml of the upper aqueous phase was mixed with 4 ml of scintillation cocktail and counted by a scintillation counter for 5 min. Protein concentrations in

these membrane preparations were measured using the BCA protein assay kit (Pierce Chemical, Rockford, IL).

Radioimmunoassay of hormones:

Plasma concentrations of oxytocin, ACTH, and corticosterone were determined by radioimmunoassays as described previously (Li, et al., 1993;Li, et al., 1997;Li, et al., 1999).

Immunoblot analyses of RGS7, pJAK2, and JAK2 proteins

Tissue preparation:

Frontal cortex from the treatment groups that received 1 mg/kg DOI challenge was used for the measurement of pJAK2, JAK2 and RGS7 proteins. Tissue was homogenized in 20 volume of ice-cold homogenization buffer which contained 25 mM HEPES-Tris, pH 7.4 at 25 °C, 1 mM EGTA and protease inhibitor cocktail (1:1000) from Sigma-Aldrich (St. Louis, MO) by using a Tekmar Tissumizer (Cincinnati, OH). The homogenate was centrifuged at 20,000 × g for 10 minutes at 4 °C. After centrifugation, the supernatant was collected as the cytosolic fraction. The tissue pellet was resuspended in 20 volumes homogenization buffer and centrifuged again. The final membrane pellet was resuspended and sonicated in 25 mM HEPES-Tris buffer containing 3 mM EGTA and 10 mM LiCl. Protein

concentrations were determined by using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

Western analyses:

Equal amounts of protein from vehicle-control and drug-treated samples were separated on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membrane for 2 hr at 100V. Non-specific binding was blocked either in TBS containing 5% (w/v) nonfat dry milk with 0.1% Tween 20 (TBST) or in PBS containing 5% (w/v) nonfat dry milk. The following primary antibodies were used: anti-RGS7 (Polyclonal antibody, Upstate Biotechnology, Inc., Lake Placid, NY), anti-phospho-JAK2 (polyclonal antibody, Affinity Bioreagent, CO), anti-JAK2 (polyclonal antibody, Upstate Biotechnology, Inc., Lake Placid, NY) and anti-actin (monoclonal antibody MP Biomedicals, Aurora, OH). Prior to incubation with a second primary antibody, blots were stripped with Restore western blot stripping buffer (Pierce, Rockford, IL) by incubating at 37^0C for 25 minutes. After incubation, blots were removed from stripping buffer, washed three times for 10 minutes each with TBS or PBS containing 0.1% Tween20 (TBST or PBST) and blocked with 5% milk in TBST or PBST for 1 hr at room temperature. Protein bands were analyzed densitometrically using Scion Image software (Scion Corporation, Frederick, MD). The gray scale density readings were calibrated using a transmission step-wedge standard. The integrated optical density (IOD) of each band was calculated as the sum of the optical densities of all

the pixels within the area of the band outlined. The IOD for the film background was subtracted from the IOD for each band. Each sample was measured in triplicate. RGS7 protein was normalized to actin protein and phosphoproteins were normalized to the corresponding total protein levels. Protein levels from treated cells were normalized to vehicle-treated cells for each western blot analysis.

RNA Isolation and Reverse Transcription:

Total RNA was isolated using the RNeasy Mini Kit (Qiagen Sciences, Valencia, CA) according to the manufacturer's protocol. Total RNA was quantitated using a spectrophotometer and optical density (OD) 260/280 nm ratios were determined. Quality of RNA was determined with a formaldehyde-agarose gel. First strand cDNA was synthesized using random hexamers and Superscript II Reverse Transcriptase from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. Reactions were incubated at 25^0C for 2 minutes, 25^0C for 10 minutes, and 42^0C for 50 minutes and inactivated by heating at 70^0C for 15 minutes in an M J Mini, personal thermal cycler (BIO-RAD, Hercules, CA).

Real-Time PCR:

The GAPDH (sense 5'-tggagtctactggcgtcttcac-3'; antisense 5'-ggcatggactgtggtcatga-3') and RGS7 (sense 5'-gaagatgagttgcaccgacaga-3'; antisense 5'-ggtctttcagtgcctcatccat-3') primer sets were synthesized by IDT,

Inc (Coralville, IA). PCR amplification was performed with 7500 Real-Time PCR System using SYBR green PCR master mix (Applied Biosystems, Foster City, CA). The PCR parameters used were a 10 minute denaturation cycle at 95°C, 40 cycles of amplification at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. Real-Time PCR was performed with 25 μL reaction mixture of cDNA, primers and SYBR green master mix.

RNA Data Analysis:

Comparative Ct (ΔΔCT) method was used for analysis of all real-time PCR data. ΔCT values were calculated by normalizing CT values of RGS7 to GAPDH from vehicle and drug-treated animal groups. The extent of the response is determined by $2^{mean(\Delta\Delta CT)}$, and the relative degree of response is calculated by $2^{-mean\ (\Delta\Delta CT)}$. Results are expressed as fold change in RGS7 mRNA levels for AG490, olanzapine, AG490 and olanzapine-treated cells with respect to vehicle-treated cells. Data presented are from four independent experiments performed in triplicate.

Statistical analyses:

All statistical analyses were performed using GB-STAT School Pak (Dynamic Microsystems, Silver Spring, MD). Data are expressed as means ± SEM. For RT-PCR, western blots data, and PLC activity assay was analyzed using a two-way analysis of variance, followed by a Newman–Keuls' post hoc

analysis. Hormone levels were analyzed using a three-way analysis of variance, followed by Newman-Keuls' post hoc analysis.

Results

Inhibition of the JAK-STAT pathway attenuated the olanzapine-induced inhibition of 5-HT_{2A} receptor-mediated PLC activity:

We previously reported that olanzapine treatment for 7 days caused a significant decrease in 5-HT-stimulated PLC activity in the rat frontal cortex (Muma, et al., 2007). Consistent with our previous report, 10mg/kg olanzapine treatment for 7 days caused a significant decrease (*$p<0.01$) in 5-HT-stimulated PLC activity (Figure 17A) in the frontal cortex. Olanzapine treatment decreased PLC activity by 68%. PLC activity was not affected by AG490 treatment alone. However, PLC activity was significantly attenuated (*$p<0.05$) in rats injected with AG490 at 10 mg/kg, before olanzapine injections, suggesting activation of the JAK-STAT pathway is necessary for the desensitization response induce by olanzapine treatment. Two-way ANOVA indicates a main effect of olanzapine ($F_{(1,19)} = 5.91$, $p<0.05$), a main effect of AG490 ($F_{(1,19)} = 46.18$, $p<0.001$), but no significant interaction was observed between olanzapine and AG490 ($F_{(1,19)} = 1.27$, $p=.275$). Furthermore, AG490 or olanzapine treatment has no effect on GTPγS-stimulated PLC activity suggesting that the desensitization response is occurring at 5-HT_{2A} receptor or 5-HT_{2A} receptor-$G_{\alpha q/11}$ interface (figure 17B).

Phosphorylation of JAK2 was blocked by AG490 treatment:

Treatment with olanzapine for 7 days significantly increased (*p<0.05) phosphorylation of JAK2 in the membrane fraction of the frontal cortex as shown in figure 18. The total JAK2 protein level was not significantly altered with olanzapine treatment. The olanzapine-induced increases in phosphorylation of JAK2 were significantly reduced to basal levels by pre-injections of AG490 at 10 mg/kg. AG490 alone had no effect on JAK2 phosphorylation. Two way ANOVA indicates a main effect of olanzapine ($F_{(1,15)}$ = 19.88, p < 0.001), a main effect of AG490 ($F_{(1,15)}$ = 37.41, p < 0.001), and a significant interaction between olanzapine and AG490 was also observed ($F_{(1,15)}$ = 31.28, p = 0.001).

Increase in RGS7 mRNA and protein levels by olanzapine treatment was blocked by pre-treatment with AG490:

Olanzapine injections for 7 days significantly increased mRNA (*p<0.05) and protein (*p<0.05) levels of RGS7 as shown in figure 19A and 19B. Both message and protein levels of RGS7 were not affected by AG490 treatment alone. In rats pre-injected with AG490 at 10 mg/kg, the olanzapine-induced increases in RGS7 expression were significantly attenuated to basal levels. Two-way ANOVA for mRNA measurement indicates a main effect of olanzapine ($F_{(1,15)}$=43.43, P<0.001), a main effect of AG490 ($F_{(1,15)}$=155.96, P<0.001), and a significant interaction between olanzapine and AG490

($F_{(1,15)}$=69.51, P<0.001). Two-way ANOVA for protein levels indicate a main effect of olanzapine ($F_{(1,15)}$=10.30, P<0.01), a main effect of AG490 ($F_{(1,15)}$=12.30, P<0.01), and a significant interaction between olanzapine and AG490 ($F_{(1,15)}$=12.40, P<0.01).

Effect of olanzapine on plasma levels of oxytocin, ACTH, and corticosterone

Basal plasma ACTH, corticosterone, and oxytocin levels were not significantly altered after seven daily injections of AG490 and olanzapine. An acute dose (i.e., a challenge dose) of DOI was administered 30 minutes before the measurement of plasma hormone levels.

Oxytocin response to DOI:

DOI-challenge produced a significant (*p<0.01) increase in plasma oxytocin levels (1257%) in vehicle-pretreated rats, in 2 mg/kg AG490 injected rats increase (853%) (*p<0.01), and in 10 mg/kg AG490 injected rats (449%) (*p<0.01) compared to saline-challenged rats. However, there were significant differences in the DOI-stimulated increases in plasma oxytocin, suggesting a dose-dependent inhibition on oxytocin release with AG490 treatment. Olanzapine treatment completely blocked the DOI-induced increases in oxytocin release as compared to vehicle-treated rats (Figure 20A). Although, a trend towards reversal of olanzapine-inhibited oxytocin level was observed at high dose of AG490 (10 mg/kg), it was statistically insignificant. Three-way ANOVA indicates a main effect of DOI challenge on oxytocin levels ($F_{(1,81)}$=

44.62, P<0.0001), a main effect of olanzapine ($F_{(1,81)}$= 5.36, P<0.05), and a main effect of AG490 ($F_{(2,81)}$= 9.94, P<0.001). Significant interactions between DOI X olanzapine ($F_{(1,81)}$= 28.12, P<0.0001) , DOI X AG490 ($F_{(2,81)}$= 63.57, P<0.0001), and olanzapine X AG490 ($F_{(2,81)}$= 165.69, P<0.0001) were also observed. Finally, there was significant interaction among DOI X olanzapine X AG490 ($F_{(2,81)}$= 65.83, P<0.0001).

ACTH response to DOI:

DOI-challenge produced a significant (*p<0.01) increase in plasma ACTH levels (1279%) in control rats, in 2 mg/kg AG490 injected rats (*p<0.01) (1296%), and in 10 mg/kg AG490 injected rats (*p<0.01) (1032%) compared to vehicle-treated rats. Olanzapine treatment significantly (38%,*p<0.01) attenuated the DOI-stimulated increase in ACTH release as compared to vehicle treated rats (Figure 20B). However, AG490 treatment had no significant effect on ACTH levels in rats treated with olanzapine compared to vehicle-treated rats. Three-way ANOVA indicates a main effect of DOI challenge on ACTH levels ($F_{(1,66)}$= 118.44, and a main effect of AG490 ($F_{(2,66)}$= 22.32, P<0.0001). A significant interaction between DOI X olanzapine ($F_{(1,66)}$= 16.46, P<0.001) , DOI X AG490 ($F_{(2,66)}$= 99.68, P<0.0001), and olanzapine X AG490 ($F_{(2,66)}$= 1897.87, P<0.0001) was also observed. There was also a significant interaction among DOI X olanzapine X AG490 ($F_{(2,66)}$= 155.32, P<0.0001).

Corticosterone response to DOI:

DOI-challenge produced a significant (*p<0.01) increase in plasma corticosterone levels (685%) in vehicle-pretreated rats, in rats treated with 2 mg/kg AG490 (524%) (*p<0.01), and in 10 mg/kg AG490 injected rats (406%) (*p<0.01) compared to saline-challenged rats. Olanzapine treatment significantly (54%,*p<0.01) attenuated the DOI stimulated increase in corticosterone release as compared to vehicle treated rats (Figure 20C). However, AG490 pretreatment had no significant effect on corticosterone levels in rats treated with olanzapine. Three-way ANOVA indicates a main effect of DOI challenge on ACTH ($F_{(1,82)}$= 43.57, P<0.0001), a significant effect of olanzapine ($F_{(1,82)}$= 5.96, P<0.001), a main effect of AG490 ($F_{(2,82)}$= 10.55, P<0.0001). A significant interaction between DOI X olanzapine ($F_{(1,82)}$= 29.63, P<0.001) , DOI X AG490 ($F_{(2,82)}$= 62.98, P<0.0001), and olanzapine X AG490 ($F_{(2,82)}$= 164.50, P<0.0001) was also observed. Finally, there was a significant interaction between DOI X olanzapine X AG490 ($F_{(2,82)}$= 68.01, P<0.0001).

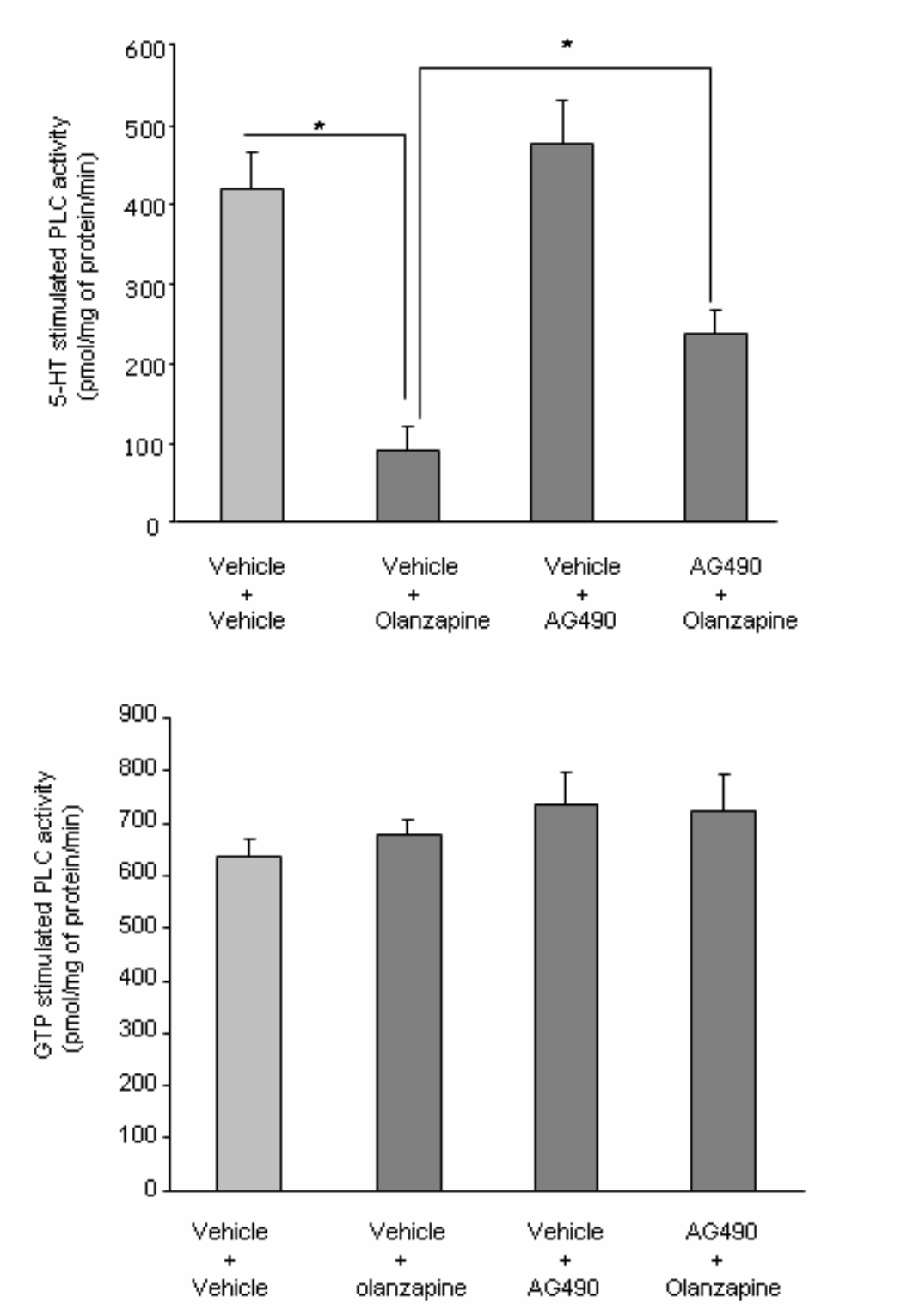

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Figure 17. PLC activity in the frontal cortex. (A): 5-HT-stimulated PLC activity in the frontal cortex is significantly decreased with 7 days of daily olanzapine injections compared to vehicle-treated control rats (*$p < 0.01$). AG490 injections alone had no effect on the PLC activity whereas the olanzapine-induced decrease in PLC activity was significantly (*$p<0.05$) attenuated by pre-injections of AG490 (10 mg/kg). (B) GTPγS-stimulated-PLC activity was not altered by either the AG490 or olanzapine treatments.

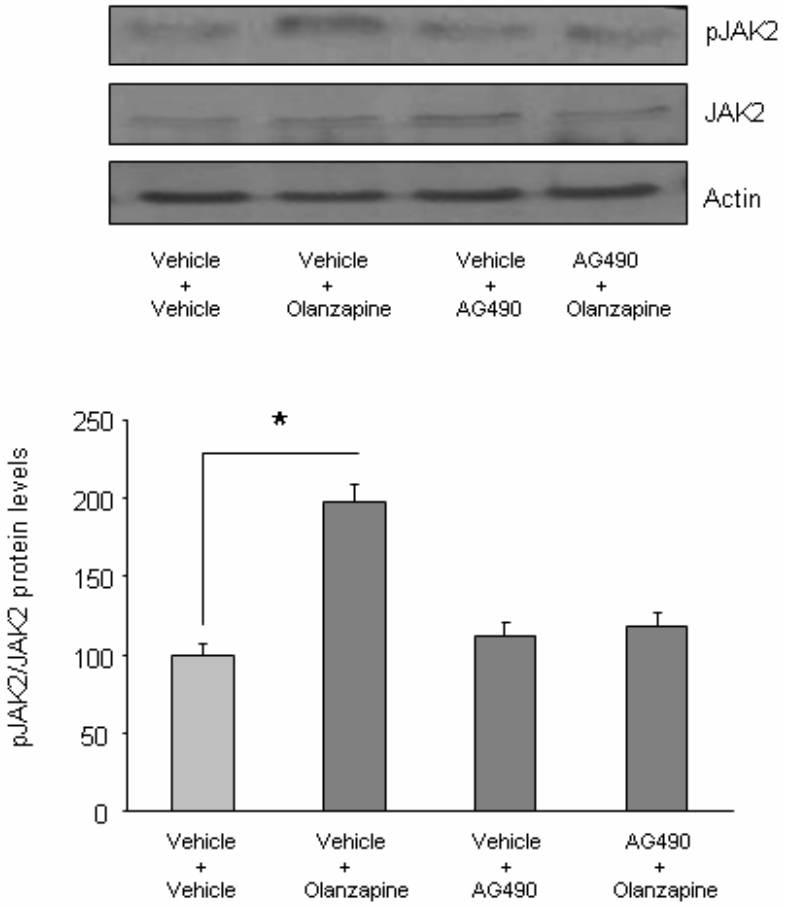

Figure 18. Olanzapine treatment significantly increased (* $p<0.05$) phosphorylation of JAK2 in the frontal cortex. AG490 treatment alone had no effect on phosphorylation of JAK2, however, inhibition of the JAK-STAT pathway with AG490 pre-treatment significantly attenuated phosphorylation of JAK2. Total JAK2 protein levels were not altered. (* indicate significantly different from olanzapine treated rats $p<0.05$), whereas AG490 alone had no effect on JAK2 levels.

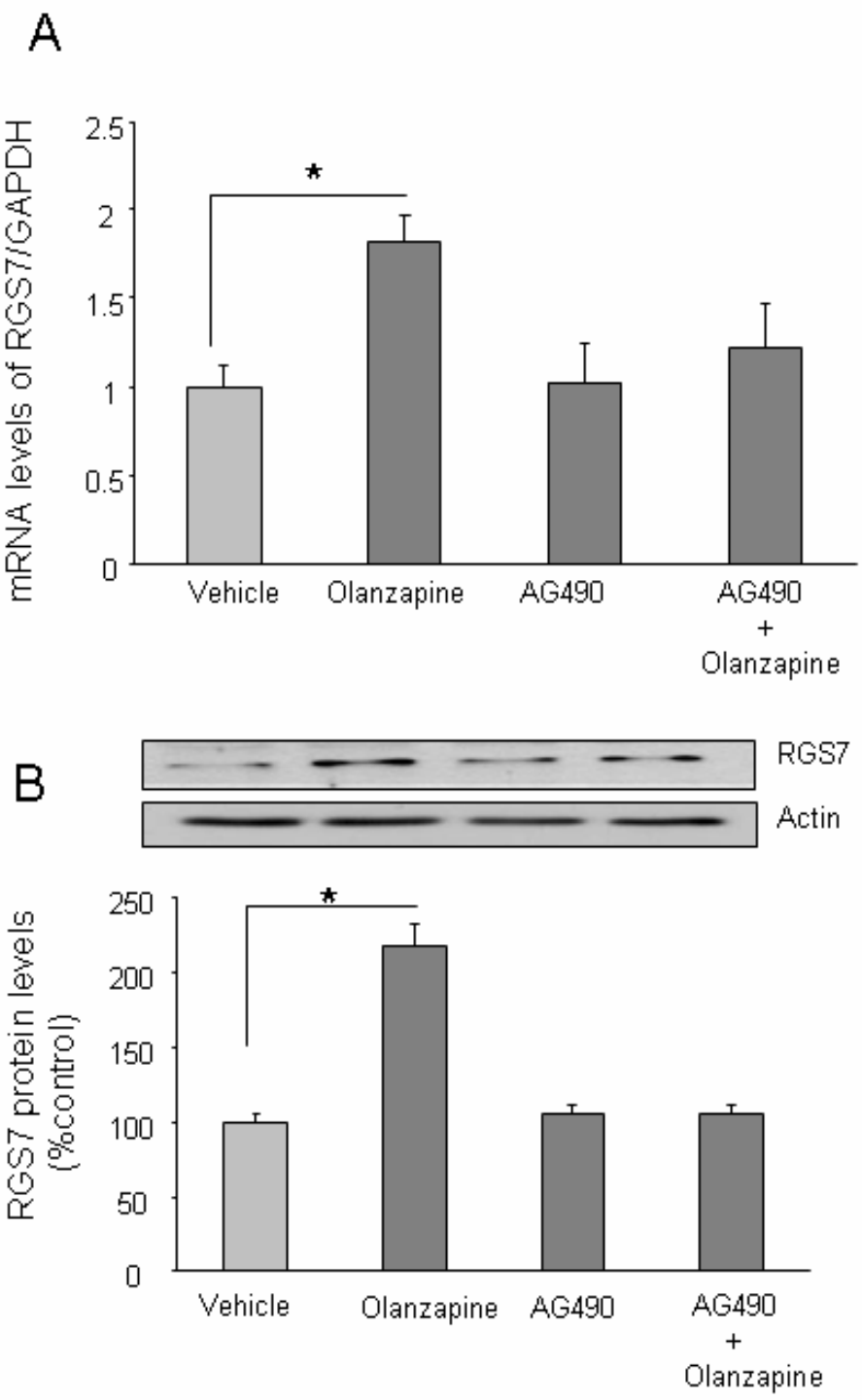

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Figure 19. Olanzapine treatment significantly (* $p<0.05$) increased RGS7 mRNA in the frontal cortex (A) and membrane-bound RGS7 protein levels (B) over vehicle-treated control rats. AG490 pre-treatment completely blocked the olanzapine-induced increase in mRNA levels of RGS7. Olanzapine-induced increase in protein levels were also blocked by AG490 pretreatment. (* indicate significantly different from olanzapine treated rats $p<0.05$), whereas AG490 alone had no effect on RGS7 levels.

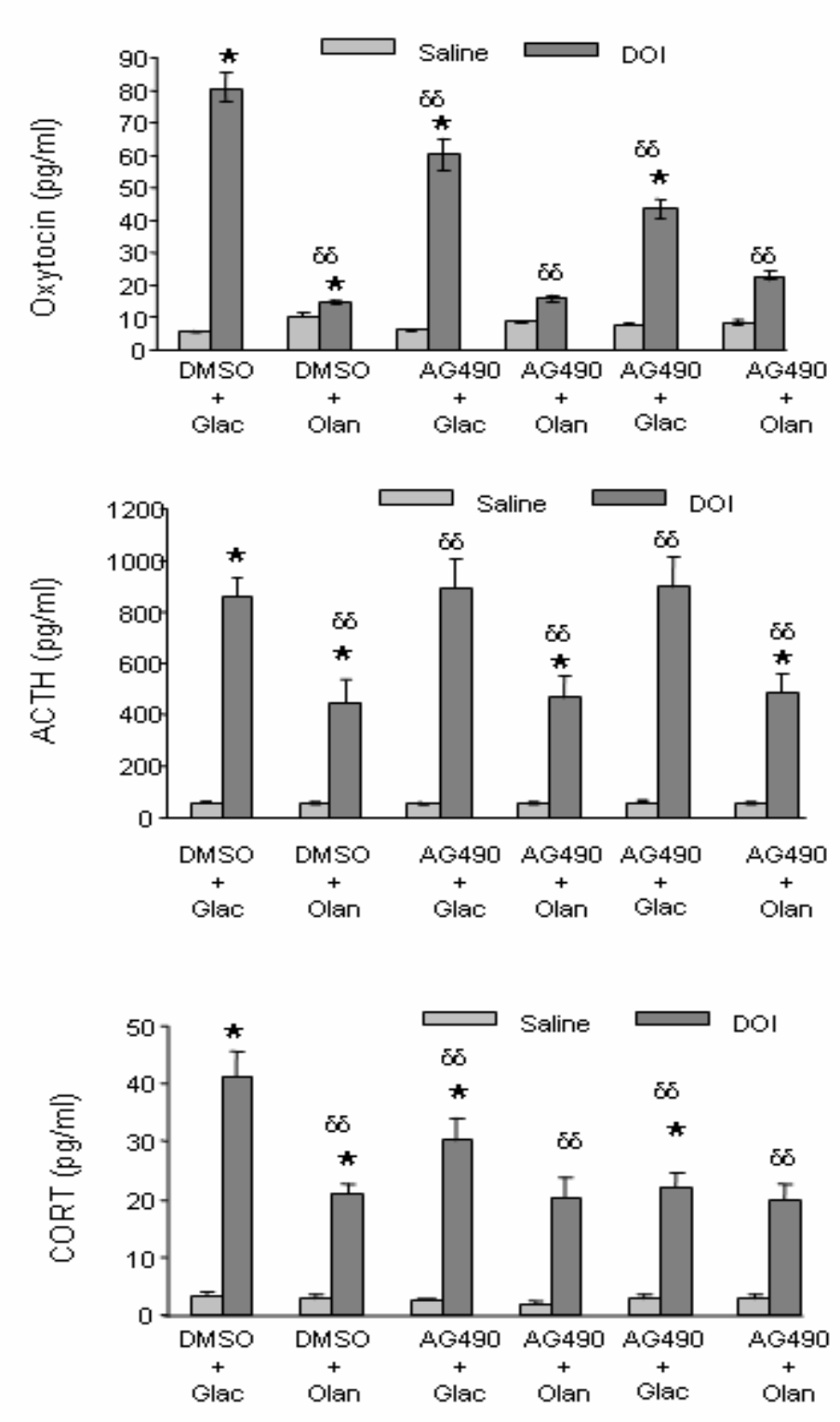

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Figure 20. Chronic treatment with olanzapine significantly attenuated DOI-stimulated hormone responses. Oxytocin (A), ACTH (B) and corticosterone (C) responses to a challenge with DOI 1 mg/ml at 30 min post-injection. The data represent the mean ± S.E.M. of eight rats per group. Basal plasma oxytocin, ACTH and corticosterone levels were not significantly different among vehicle, AG490 and olanzapine injected rats. DOI-challenge induced a significant increase of plasma oxytocin, ACTH and corticosterone levels compared with saline-challenge groups. A significant difference amongst DOI-challenged control and treated rats is indicated by * for $p < 0.001$. A significant effect of chronic olanzapine and AG490 treatment compared with vehicle treatment is indicated by $\delta\,\delta$, $p < 0.01$.

Discussion

Although, atypical antipsychotics bind to diverse population of receptors (Meltzer, et al., 1989;Altar, et al., 1986;Zhang and Bymaster, 1999;Roth, et al., 2004), the therapeutic benefits associated with atypical antipsychotics are attributed, in part, to their ability to antagonize 5-HT_{2A} receptor signaling (i.e. desensitization) (Goyer, et al., 1996). Previous studies have shown atypical antipsychotics induced desensitization of 5-HT_{2A} receptor signaling both in vivo and in cells (Gray and Roth, 2001;Hanley and Hensler, 2002). Internalization and down-regulation are suggested to be involved in the desensitization response; however, the underlying molecular mechanisms necessary for desensitization are not yet identified. Previously, we reported that both in rat frontal cortex and in cells in culture, olanzapine causes desensitization of 5-HT_{2A} receptor signaling, increases RGS7 expression and activates the JAK-STAT pathway (Muma, et al., 2007;Singh, et al., 2007).

Consistent with our previous findings in cultured A1A1v cells, we now find that the olanzapine-induced decrease in 5-HT_{2A} receptor mediated-PLC activity in the rat frontal cortex is dependent in part on activation of the JAK-STAT pathway. Olanzapine treatment increases RGS7 mRNA and protein levels. We previously reported that increased RGS7 protein expression in response to olanzapine treatment is dependent on JAK-STAT signaling in A1A1v cells (chapter I). Consistent with this report, increases in RGS7 mRNA and protein levels are blocked by inhibition of JAK-STAT pathway in frontal

cortex. The olanzapine-induced desensitization of 5-HT_{2A} receptor signaling was significantly attenuated with pretreatment with a JAK2 kinase inhibitor. These data suggest that activation of the JAK-STAT pathway is necessary for olanzapine-induced desensitization of 5-HT_{2A} receptor signaling.

RGS proteins can reduce G-protein-mediated signaling by their ability to increase the intrinsic guanosine triphosphatase (GTPase) activity of heterotrimeric G proteins. This GTPase accelerating protein (GAP) activity enhances G protein deactivation and promotes desensitization (Hollinger and Hepler, 2002;Dohlman and Thorner, 1997). RGS7 protein is primarily expressed in the brain (Koelle and Horvitz, 1996;Saitoh, et al., 1999). The expression of RGS7 mRNA in the brain is widespread and abundant. An increase in RGS7 protein following olanzapine treatment would increase the termination rate of 5-HT_{2A} receptor-$G_{\alpha q/11}$ protein signaling by more rapidly hydrolyzing GTP, and could thereby produce or contribute to the desensitization response. When GTPγS, a non hydrolysable GTP analogue, is used to activate G-proteins, PLC activity is not affected by RGS proteins, since RGS proteins are not able to hydrolyze GTPγS bound to G proteins. Therefore, the differential effects of olanzapine on receptor versus G-protein activation of PLC activity are consistent with an increase in RGS7 protein as an underlying mechanism for olanzapine-induced desensitization. The increased levels of RGS7 proteins could reduce 5-HT-stimulated PLC activity as we found in olanzapine-treated cell membranes; however, GTPγS

stimulated-PLC activity was not altered by olanzapine consistent with increased RGS proteins underlying the desensitization response. These results are consistent with our previous report where AG490 pretreatment significantly attenuated the olanzapine-induced decrease in 5-HT_{2A} receptor-mediated PLC activity in A1A1v cells but GTPγS stimulated-PLC activity was not altered by olanzapine.

Previous studies have reported down-regulation of RGS4 expression in the frontal cortex of schizophrenic patients (Mirnics et al., 2001; Gu et al., 2007). Although, none of these studies have identified alterations in RGS7 expression. Since RGS7 and RGS4 are independently regulated (Krumins et al., 2004), the olanzapine-induced increase in RGS7 protein might replace the diminished RGS4 levels and thereby restore the 5-HT_{2A} receptor signaling duration to more physiological levels.

The olanzapine-induced increase in RGS7 levels could be mediated by either increased stability of RGS7 proteins and/or increased transcription of RGS7 mRNA. Interaction of RGS7 with Gβ5 (Zhang and Simonds, 2000) and polycystine (Kim, et al., 1999) is reported to increase the stability of RGS7. In addition, interaction of RGS7 with 14-3-3 is reported to control its GAP activity (Benzing, et al., 2000). However, our real-time PCR data suggest that the increase in RGS7 levels by olanzapine is mediated by a direct increase in RGS7 mRNA via activation of JAK-STAT pathway. In addition, previous reports have suggested that activation of JAK-STAT by tumor necrosis factor

α (TNFα) (Guo, et al., 1998) decreases the inhibitory interaction of RGS7 with 14-3-3 thereby increasing its GAP function (Benzing, et al., 2002). Thus, it is possible that activation of JAK-STAT pathway by olanzapine treatment could increase GAP activity of RGS7 by inhibiting its interaction with 14-3-3.

In our previous studies we identified a STAT3 consensus binding elements located 2.34kb upstream of transcription start site that binds strongly with STAT3 in response to olanzapine treatment. Thus, STAT3 binding to the RGS7 gene along with an increase in mRNA levels of RGS7 suggest the possibility that STAT3 could be a transcription factor for RGS7. Taken together, these results are consistent with our hypothesis that atypical antipsychotics increase RGS7 expression via activation of the JAK-STAT pathway.

While the precise mechanism of JAK-STAT activation by atypical antipsychotics is not determined, it has been reported that the 5-HT_{2A} receptor associates in a complex with JAK2 and STAT3 (Guillet-Deniau, et al., 1997). Other studies have reported that 5-HT activates JAK2, JAK1, and STAT1 via the 5-HT_{2A} receptors (Banes, et al., 2005). Furthermore, atypical antipsychotics have also been reported to activate other signaling cascades for example, activation of ERK1/2 pathways in the rat frontal cortex (Fumagalli, et al., 2006), Akt/PKB and P38 pathways in PC12 cells (Lu, et al., 2004), and GSK3α/β in the rat frontal cortex (Kang, et al., 2004;Roh, et al., 2007). It is also important to emphasize that these studies were conducted in

different experimental settings with different time course and dose regimens than our study. Each study highlights important findings, further extending our understanding of the mechanisms of action of antipsychotics. It is becoming evident that there could be numerous pathways and alterations in gene expression that lead to the development of psychosis and its treatment with atypical antipsychotics. We found almost 50% recovery of the olanzapine-induced reductions in PLC activity with inhibition of the JAK-STAT pathway, suggesting one of these other signaling cascades together with the JAK-STAT pathway could participate in mediating desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics. Similarly, we previously found that inhibition of the JAK-STAT pathway reduced by the olanzapine-induced desensitization response by approximately half in A1A1v cells. These results produced by inhibition of the JAK-STAT pathway suggest that activation of the JAK-STAT pathway is necessary but not sufficient to induce complete desensitization of 5-HT_{2A} receptor signaling by atypical antipsychotics and may involve other signaling cascades.

A wide range of studies have suggested a link between hypothalamic-pituitary-adrenal (HPA)-axis dysfunction and psychiatric disorders particularly negative symptomatology in schizophrenia (Shirayama, et al., 2002;Walker, et al., 2002). Subsequent studies have demonstrated that the atypical antipsychotic- induced decrease in cortisol levels is associated with an improvement in psychopathology (Meltzer, 1989;Hatzimanolis, et al.,

1998;Markianos, et al., 1999). The olanzapine-induced decrease in 5-HT_{2A} receptor stimulated plasma levels of oxytocin, ACTH, and corticosterone in this study are consistent with previous reports (Scheepers, et al., 2001;Wik, 1995;Tepavcevic, et al., 1994) . However, unlike in the frontal cortex where preinjections of AG490 significantly attenuated the olanzapine-induced decrease in 5-HT_{2A} receptor-mediated PLC activity, olanzapine-induced desensitization of 5-HT_{2A} receptor stimulated plasma hormone release was not affected by inhibition of JAK-STAT pathway.

We did however find a selective dose-dependent inhibitory effect of AG490 on 5-HT_{2A} receptor stimulated oxytocin and corticosterone levels whereas ACTH levels were not affected. Previous studies have reported that corticotropin releasing hormone (CRH) and oxytocin-containing neurons in the PVN, are directly innervated by 5-HT containing nerve terminals originating in the dorsal raphe nucleus (Liposits, et al., 1987;Sawchenko, et al., 1983;Kawano, et al., 1992;Saphier, 1991). Subsequent reports have further demonstrated the presence of 5-HT_{2A} receptors on the oxytocin and CRH neurons in the PVN. Treatment with MDL100907, a specific antagonist of 5-HT_{2A} receptor, inhibited release of oxytocin and ACTH in response to DOI, a 5-$HT_{2A/2C}$ agonist (Damjanoska, et al., 2003;Zhang, et al., 2004). However, it is not clear how inhibition of the JAK-STAT pathway independently regulates release of oxytocin and corticosterone. Previous studies investigating effect of clozapine on inhibition of CRH gene

transcription, suggested involvement of various kinases like PI3K, PKA, PKC, CaMK, and MAPK, Akt (Basta-Kaim, et al., 2006). This is consistent with the ability of atypical antipsychotics to induce alterations in gene expression probably by activation of various signaling cascades (Feher, et al., 2005;Fatemi, 2006). Thus, olanzapine-induced activation of JAK-STAT pathway together with other signaling cascades may affect the gene expression of oxytocin and CRH thereby decreasing their plasma levels independent of the olanzapine response. Furthermore, as we have previously noted for PLC activity in A1A1v cells (Singh, et al., 2007), AG490 may interfere with the release of these hormones in the blood stream.

Taken together, this study further confirmed that olanzapine-induced desensitization of 5-HT_{2A} receptor-mediated PLC activity in frontal cortex is dependent in part on increased expression of RGS7 via activation of the JAK-STAT pathway. Our plasma hormone data confirmed that olanzapine causes desensitization of 5-HT_{2A} receptors in the hypothalamus but further studies are needed to determine the role of JAK-STAT pathway in the regulation of hormone levels.

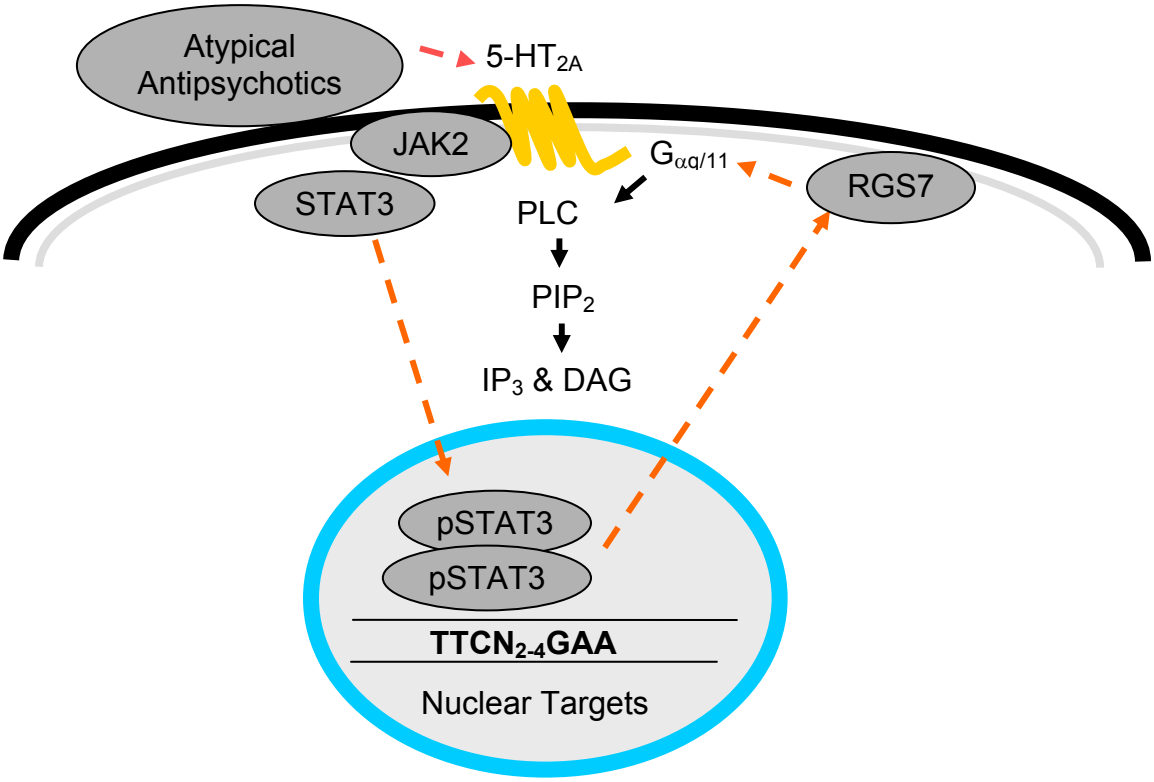
CHAPTER VI

CONCLUSIONS

Our results suggest that atypical antipsychotics desensitize 5-HT_{2A} receptor signaling both in cells and in vivo by activation of the JAK-STAT pathway. Phosphorylated and activated STAT3 forms a homodimer and translocates to the nucleus (figure 21), as suggested by increased phospho-STAT3 levels in the nucleus. In the nucleus, phospho-STAT3 binds to its consensus element ($TTCN_{2-4}GAA$) on the RGS7 putative promoter region at 2.34kb upstream from the transcription start site. Treatment with atypical antipsychotics also increased RGS7 mRNA and proteins levels, which were blocked by pretreatment with a selective JAK2 inhibitor, AG490, suggesting that the increase in RGS7 expression is dependent on activation of the JAK-STAT pathway. Furthermore, an increase in RGS7 protein following atypical antipsychotic treatment could increase the termination rate of 5-HT_{2A} receptor- $G_{\alpha q/11}$ protein signaling by more rapidly hydrolyzing GTP, and could thereby produce or contribute to the desensitization response. In addition, inhibition of the JAK-STAT pathway also significantly reduced the desensitization response induced by atypical antipsychotics as measured by 5-HT_{2A} receptor-mediated PLC activity, suggesting that desensitization is dependent on activation of the JAK-STAT pathway. Although atypical antipsychotics bind with high affinity to a number of receptors, MDL100907 also increased RGS7 mRNA and protein levels and activation of the JAK-

STAT pathway in our cell culture model suggesting that antagonism at 5-HT_{2A} receptors is sufficient for induction of these changes. The results of the present studies support our hypothesis that atypical antipsychotic-induced desensitization of 5-HT_{2A} receptor signaling is dependent on activation of the JAK-STAT pathway and subsequent increases in RGS7 expression.

In the present study, we found almost 50% recovery of the olanzapine-induced reductions in PLC activity with inhibition of the JAK-STAT pathway, suggesting that activation of the JAK-STAT pathway is necessary but not sufficient for olanzapine-induced desensitization. It has been reported that another tyrosine signaling pathway ERK1/2 and serine/threonine signaling proteins like (GSK)-3β are activated by stimulation of 5-HT_{2A} receptors and atypical antipsychotics like olanzapine (Browning, et al., 2005;Quinn, et al., 2002;Roh, et al., 2007). Thus, it is likely that atypical antipsychotic-induced desensitization of 5-HT_{2A} receptor signaling could be in part mediated by these other signaling pathways. This can be tested by monitoring 5-HT_{2A} receptor-mediated PLC activity in the presence of a selective ERK1/2 pathway inhibitor SB386023 and/or (GSK)-3β inhibitor SB-216763 separately and along with AG490. If the PLC activity is completely restored by a combination of these inhibitors with AG490, this would confirm that desensitization of 5-HT_{2A} receptor signaling is mediated by activation of the JAK-STAT pathway together with other signaling pathways.



<u>Figure 21:</u> Proposed model for desensitization of 5-HT$_{2A}$ receptor signaling by atypical antipsychotics: Treatment with atypical antipsychotics causes desensitization of 5-HT$_{2A}$-G$_{\alpha q/11}$ pathway as measured by either production of inositol phosphate in cells or PLC activity in brain tissue. We hypothesized that atypical antipsychotics through 5-HT$_{2A}$ receptor antagonism activate the JAK-STAT pathway causing phosphorylated STAT3 to dimerize and translocate to the nucleus, stimulate immediate early genes and subsequently increase RGS7 transcription and RGS7 protein levels in the membrane. An increase in RGS7 proteins following atypical antipsychotics treatment would increase the termination rate of 5-HT$_{2A}$ receptor-G$_{\alpha q/11}$

protein signaling by more rapidly hydrolyzing GTP, and could thereby produce or contribute to the desensitization response.

Our results are consistent with the notion that there could be numerous pathways and alterations in gene expression that are altered by treatment with atypical antipsychotics (Ko, et al., 2006). We also found that treatment with atypical antipsychotics increased RGS7 mRNA and protein levels in cells and in vivo is dependent on activation of the JAK-STAT pathway. We proposed that increases in membrane bound RGS7 protein can accelerate hydrolysis of activated $G_{\alpha q/11}$ that results in desensitization of 5-HT_{2A} receptor signaling. RGS7 like other regulatory proteins is an unstable protein and is rapidly degraded by the ubiquitin−proteasome pathway. Thus, it would be important to measure the GAP activity and half-life of RGS7 proteins to further confirm that the atypical antipsychotic-induced increase in RGS7 expression is indeed occurs. The GAP activity of RGS7 can be measured by multiple mechanisms as reported in literature such as an in vitro GAP assay using radioisotope ([γ-^{32}P] GTP) (Shuey, et al., 1998) and a direct fluorescence-based assay for RGS domain GAP activity (Willard, et al., 2005). In addition, it has been extensively reported that interaction with proteins such as polycystin, $G_{\beta 5}$, and 14-3-3 regulate the stability and activity of RGS7 proteins. RGS7 contains a putative coiled-coil structure flanked by two potential PEST sequences (Rechsteiner, 1990) that contain minimal

consensus phosphorylation sites necessary for degradation. Polycystin by directly binding to a PEST sequence-containing domain of RGS7 inhibits the phosphorylation-dependent ubiquitination and subsequent degradation of RGS7 and thereby modulate its functional activity. It has also been suggested that polycystin may relocalize RGS7 to a subcellular compartment away from ubiquitin-protein ligases responsible for recognition and targeting of RGS7 to the proteasome (Kim, et al., 1999). In addition, association of RGS7 with $G_{\beta 5}$ is reported to stabilize the protein and enhance the GAP function of RGS7 on acceleration of GIRK channel kinetics (Kovoor, et al., 2000).

Furthermore, RGS7 contains a functional 14-3-3-binding site within the RGS domain and a significant fraction of RGS7 normally exists bound to endogenous 14-3-3. The binding of RGS7 to 14-3-3 is phosphorylation-dependent; the primary 14-3-3-binding site in RGS7 involves serine 434, a region implicated in interactions with G_{α} subunits. It has been reported that phosphorylation and subsequent interaction with 14-3-3 results in a progressive decline in the GAP activity of RGS7 proteins (Benzing, et al., 2000). Interestingly, previous reports have suggested that tumor necrosis factor α (TNF) by binding to its receptor TNFR1 induces activation of the JAK-STAT pathway (Guo, et al., 1998). Additionally, it has been demonstrated that treatment of mice with TNF-α inhibits the phosphorylation of serine 434 and completely abrogated the interaction of RGS7 with 14-3-3. In fact, RGS-mediated deactivation kinetics of G protein-coupled inwardly

rectifying K+ channels (GIRKs) in a Xenopus co-expression system was slowed by microinjections of 14-3-3 protein (Benzing, et al., 2002).

Thus, it would be interesting to know if atypical antipsychotic-induced activation of the JAK-STAT pathway affects interactions of RGS7 with any of these proteins thereby affecting its GAP activity and stability. Although, a RGS7 knock-out animal model is not available, using a siRNA approach to decrease RGS7 expression, it can be determined whether increased RGS7 is necessary for atypical antipsychotics-induced desensitization of 5-HT_{2A} receptors signaling in cells. This can be further examined in animals using a viral vector to deliver siRNA in the brain.

In the present study, we reported that treatment with olanzapine increases STAT3 binding to its consensus element at 2.34kb upstream from transcription start site on the putative RGS7 promoter region. STAT3 binding to the putative RGS7 promoter region can be further verified with electro-mobility shift assay (EMSA). The advantages of studying DNA: protein interactions by EMSA are that the source of the DNA-binding protein may be a crude nuclear or whole cell extract rather than a purified preparation. Gel shift assays can be used qualitatively to identify sequence-specific DNA-binding proteins (such as STAT3) in crude lysates. Although, transcriptional activity of STAT3 has been extensively reported for various genes (Aaronson and Horvath, 2002;Kisseleva, et al., 2002;Schindler, 2002), STAT3 has not been identified as a transcription factor for RGS7. STAT3-mediated regulation

of gene expression is associated with the presence of the consensus element $TTCN_{2-4}GAA$ upstream of the transcription start site (Ehret, et al., 2001;Wrighting and Andrews, 2006). Genomic sequence analysis of rat RGS7 revealed that there are multiple sets of $TTCN_{2-4}GAA$ sequences. Thus, it is possible that STAT3 is a transcription factor for the RGS7 promoter. The promoter region of RGS7 is not yet identified, however, it is usually present upstream of the transcription start site consistent with our identified STAT3 binding site 2.34kb upstream from the transcription initiation site. We can further confirm the transcriptional activity of STAT3 on RGS7 promoter by using a reporter gene assay. This can be done by linking the identified putative promoter sequence to an easily detectable reporter gene such as that encoded for the firefly luciferase. Although, STAT3 knock-out is lethal, a conditional knock-out can be used to further confirm the role of STAT3 in regulating RGS7 expression.

A previous study has examined the impact of olanzapine on changes in gene expression in the frontal cortex of rats treated with olanzapine (2 mg/kg per day for 21 days) (Fatemi, et al., 2006). In this study, 31 genes were down-regulated and 38 genes were found to be upregulated including one RGS protein, RGS19. RGS7 was not identified as one of the upregulated genes in this study as expected since in our previous study, we did not find a significant increase in RGS7 protein levels with the 2 mg/kg dose of olanzapine (Muma, et al., 2007). Thus, a microarray study with a higher dose

of olanzapine (10mg/kg) based on our previous results (chapter V) can be useful in identifying changes in gene expression with chronic olanzapine treatment. The use of a higher dose of olanzapine is also supported by its half-life which in rat brain is 2.5 to 5.1 hours, much longer than the 20 to 54 hour half-life of olanzapine in humans (Aravagiri, et al., 1999). Based on our present findings, we may identify alterations in one or more proteins in the 5-HT_{2A} receptor signaling pathway by olanzapine and possibly regulated by STAT3.

In the present study, we found that treatment with olanzapine did not affect the basal levels of oxytocin, ACTH and corticosterone but decreased DOI-stimulated release of oxytocin, ACTH, and corticosterone which is consistent with previous reports (Scheepers, et al., 2001;Wik, 1995;Tepavcevic, et al., 1994). However, unlike in the frontal cortex where preinjections of AG490 significantly attenuated the olanzapine-induced decrease in 5-HT_{2A} receptor-mediated PLC activity, olanzapine-induced desensitization of 5-HT_{2A} receptor stimulated plasma hormone release was not affected by inhibition of the JAK-STAT pathway. We did however find a selective dose-dependent inhibitory effect of AG490 on 5-HT_{2A} receptor-stimulated oxytocin and corticosterone release whereas ACTH release was not affected. Although, JAK2 expression in the frontal cortex is almost twice the levels in the hypothalamus as reported previously (Berhow et al., 1996), dose-dependent inhibition of oxytocin and corticosterone release by AG490

suggests that JAK2 expression was not a factor in its inability to attenuate the desensitization response in the release of various hormones. Furthermore, the effect of JAK-STAT inhibition on 5-HT_{2A} receptor-stimulated release of oxytocin and corticosterone suggests that the JAK-STAT may be directly involved in release of these hormones. Since, in the present study we injected AG490 systemically (s.c), a direct injection locally in the PVN would provide further details of JAK-STAT impact on release of these hormones.

In addition, antipsychotic drugs are reported to regulate gene expression, including those involved in regulation of hypothalamic-pituitary-adrenal (HPA) axis, whose activity is frequently disturbed in schizophrenic patients. Recently, it has been reported that a clozapine-induced decrease in basal activity of the CRH promoter in Neuro-2A cells stably transfected with hCRH-CAT reporter construct is dependent on the activation of PI3-K/Akt pathway (Basta-Kaim, et al., 2006). Although, this report does highlight a role of signaling cascades on CRH promoter activity, it is not yet understood how treatment with atypical antipsychotics decrease cortisol levels in schizophrenic patients (Scheepers, et al., 2001;Wik, 1995;Tepavcevic, et al., 1994). Furthermore, in contrast to this study, we did not find any effect of JAK-STAT activation on ACTH release, which is regulated by CRH. In fact, we found a dose-dependent inhibitory effect of AG490 on oxytocin and corticosterone release. Since STAT3 has been extensively characterized as transcription factor for variety of genes, it would be interesting to investigate if

oxytocin and corticosterone regulating genes also contain STAT3 consensus elements. Since, both clozapine and olanzapine have similar chemical structure (thienobenzodiazepine), it would also be important to determine the role of activation of the JAK-STAT pathway in the desensitization response induced by atypical antipsychotics, by using a compound with a different chemical structure such as risperidone which contains benzisoxazole and piperidine functional groups.

Overall, these results suggest that atypical antipsychotic (thienobenzodiazepine) treatment desensitizes 5-HT_{2A} receptor function and increases expression of RGS7 mRNA and protein levels. Antagonism of 5-HT_{2A} receptors mediates the activation of the JAK-STAT pathway and is sufficient for subsequent increase in RGS7 mRNA and protein levels. Treatment with olanzapine increased STAT3 binding to its consensus element at 2.34kb upstream from transcription start site of RGS7 putative promoter region. Thus, STAT3 binding to the RGS7 gene along with an increase in mRNA levels of RGS7 suggest the possibility that STAT3 could be a transcription factor for RGS7.

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