Published in final edited form as:

Neuropharmacology. 2007 September ; 53(4): 552–562.

Chronic Olanzapine Activates the Stat3 Signal Transduction Pathway and Alters Expression of Components of the 5-HT_{2A} Receptor Signaling System in Rat Frontal Cortex

NA Muma, RK Singh, MS Vercillo, DN D'Souza, B Zemaitaitis, F Garcia, KJ Damjanoska, Y Zhang, G Battaglia, and LD Van de Kar

Department of Pharmacology, Loyola University Medical Center, Maywood, IL 60153

Summary

The mechanisms underlying desensitization of serotonin 2A (5-HT_{2A}) receptor signaling by antagonists are unclear but may involve changes in gene expression mediated via signal transduction pathways. In cells in culture, olanzapine causes desensitization of 5-HT_{2A} receptor signaling and increases the levels of regulators of G protein signaling (RGS) 7 protein dependent on phosphorylation/activation of the Janus kinase 2 (Jak2)/signal transducers and activators of transcription 3 (Stat3) signaling pathway. In the current study, the 5-HT_{2A} receptor signaling system in rat frontal cortex was examined following 7 days of daily treatment with 0.5, 2.0 or 10.0 mg/kg i.p. olanzapine. Olanzapine increased phosphorylation of Stat3 in rats treated daily with 10 mg/kg olanzapine and caused a dose-dependent desensitization of 5-HT_{2A} receptor-mediated phospholipase C activity. There were dose-dependent increases in the levels of membrane-associated 5-HT_{2A} receptor, G_{α11} and G_{αq} protein levels but no changes in the G_β protein levels. With olanzapine treatment, RGS4 protein levels increase in the membrane-fraction and decrease in the cytosolic fraction by similar amounts suggesting a redistribution of RGS4 protein within neurons. RGS7 protein levels increase in both the membrane and cytosolic fractions in rats treated daily with 10 mg/kg olanzapine. The olanzapine-induced increase in Stat3 activity could underlie the increase in RGS7 protein expression *in vivo* as previously demonstrated in cultured cells. Furthermore, the increases in membrane-associated RGS proteins could play a role in desensitization of signaling by terminating the activated G_{αq/11} proteins more rapidly.

Keywords

antipsychotic; serotonin 2A receptor; *in vivo*

Introduction

Adaptive changes in post-synaptic serotonin 2A/2C (5-HT_{2A/2C}) receptor signaling may underlie the mechanism of action of several drug treatments for neuropsychiatric disorder (Roth, et al., 1998). For example, several antipsychotic drugs, such as olanzapine, desensitize both 5-HT_{2A} and 5-HT_{2C} receptors (Roth, et al., 1998) while 5-HT uptake blockers (e.g., fluoxetine), can increase the maximal efficacy of 5-HT_{2A} receptor signaling (Damjanoska, et

Corresponding author: Nancy Muma, Ph.D. Professor and Chair Department of Pharmacology and Toxicology School of Pharmacy University of Kansas 1251 Wescoe Hall Drive 5064 Malott Hall Lawrence, KS 66045 nmuma@ku.edu phone: 785-864-4002 fax: 785-864-5219

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

al., 2003; Tilakaratne, et al., 1995). These adaptive changes may explain the 2-3 week delay in full symptom improvement seen with these drug treatments. However, the molecular mechanisms that underlie these adaptive changes in 5-HT_{2A} receptor signaling are not well understood.

Treatment with many 5-HT_{2A} receptor antagonists including olanzapine causes a decrease in the density of 5-HT_{2A} receptor binding sites with no change in K_D in rat frontal cortex and in cells in culture (Anji, et al., 2000; Kusumi, et al., 2000; Tarazi, et al., 2002; Willins, et al., 1999). Consistent with these findings, 5-HT_{2A} receptor antagonists including clozapine and olanzapine cause 5-HT_{2A} receptor internalization, i.e., redistribution of 5-HT_{2A} receptors from plasma membrane to within cell bodies, both *in vivo* and in cells in culture (Bhatnagar, et al., 2001; Willins, et al., 1999). Internalization of a number of receptors, including 5-HT_{1A} receptors and m1 muscarinic receptors, leads to activation of signal transduction pathways (Pierce and Lefkowitz, 2001). Sustained activation of specific intracellular signal transduction pathways, such as the Jak/Stat pathway, as would occur following internalization of 5-HT_{2A} receptors induced by chronic antagonist treatment, could then lead to changes in gene expression and long-term changes in the 5-HT_{2A} receptor signaling system. This receptor system is composed of 5-HT_{2A} receptors that are coupled via G_{αq/11} proteins to increase the activity of phospholipase C (PLC) (Roth, et al., 1998). Hydrolysis and thereby termination of 5-HT_{2A} receptor-activated G_{αq/11} protein signaling is enhanced by RGS4 and RGS7 proteins (Ghavami, et al., 2004; Shuey, et al., 1998).

The Jak/Stat pathway is activated by a number of G protein coupled receptors such as 5-HT_{2A}, β₂-adenoreceptors and angiotensin II receptors (Guillet-Deniau, et al., 1997; Ram and Iyengar, 2001). Activation of 5-HT_{2A} receptors causes a rapid and transient activation of Jak2 and Stat3 (Guillet-Deniau, et al., 1997). Serotonin stimulation also induced the co-immunoprecipitation of Stat3 with Jak2 and the 5-HT_{2A} receptor (Guillet-Deniau, et al., 1997). The Jak/Stat pathway regulates expression of a number of genes including cFos, c-Jun and c-Myc (Burysek, et al., 2002; Cattaneo, et al., 1999), transcription factors which can then stimulate expression of select genes. These transcription factors could impact directly on the expression of proteins in the 5-HT_{2A} receptor signaling system. In A1A1v cells, a cell line that constitutively expresses the 5-HT_{2A} receptor signaling system, 24-hour 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 (Singh, et al., 2007). Based on these previous studies in cell culture and as shown in Figure 1, we hypothesize that chronic treatment with a 5-HT_{2A} receptor antagonist causes alterations in the expression of RGS7 protein and activation of the Jak/Stat pathway *in vivo*.

Methods

Treatment

Male Sprague-Dawley rats (250-275 g; Harlan Laboratories, Indianapolis, IN) were housed 2 in a cage in an environment controlled for temperature, humidity, and lighting (lights on 7 am-7 pm), food and water were provided *ad libitum*. Rats were given 7 daily i.p. injections of olanzapine (0.5 mg/kg, 2.0 mg/kg, or 10 mg/kg) or saline. Olanzapine was chosen because it is clinically used in the treatment of schizophrenia, and it is useful in combination with fluoxetine for treatment-resistant depression, bipolar disorder and other mood disorders (Corya, et al., 2006; Marek, et al., 2003; Tohen, et al., 2003). The olanzapine doses were chosen based on previous data suggesting that a single injection of 2 mg/kg will result in peak concentrations in the clinically relevant range and repeated daily injections of 10 mg/kg/day will result in clinically relevant peak and trough concentrations of olanzapine (Kapur, et al., 2003). Rats were weighed every other day during the treatment period. On the 8th day, the rats were injected with either 1 mg/kg (-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl

(DOI) or saline s.c. The rats were killed 30 min. post injection by guillotine. 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.

Tissue Preparation

Membrane and cytosol samples were prepared for the PLC assay and western blot procedure as previously described (Damjanoska, et al., 2004) using tissue from rats given saline challenge injections. The membrane-bound proteins were collected for the PLC activity measurements and both membrane-bound and cytosolic protein fractions were examined by immunoblot analysis. For analysis of Stat3, tissues from the DOI-challenged rats were used and were homogenized in 10 mM Tris buffer, pH 7.7, containing 100 mM NaCl, 1 mM EDTA, and protease inhibitors purchased as a cocktail (containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, bestatin, leupeptin, and aprotinin) from Sigma (1.5μL/30mg tissue; St. Louis, MO). Phosphatase inhibitors (50 mM NaF and 1 mM Na₃VO₄) were also added to the homogenation buffer. The protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Phospholipase C_β (PLC) Activity

PLC activity was measured in membrane fractions of rat frontal cortex as previously described using an ED50 concentration of GTPγS and an Emax concentration of serotonin (Damjanoska, et al., 2003; Wolf and Schutz, 1997). 5-HT-stimulated PLC activity in the frontal cortex is almost entirely mediated by 5-HT_{2A} receptors, as pretreatment with 5-HT_{2A} receptor antagonists (ketanserin, spiperone, or mianserin) inhibits most of the 5-HT-stimulated PLC activity in the frontal cortex of rats (Wolf and Schutz, 1997). Each experiment was repeated at least three times.

Immunoblots

Immunoblot analysis of proteins has been described previously in detail (Damjanoska, et al., 2003). Briefly, the membrane or cytosolic proteins (10-2.5μg/lane depending on the antibody used) were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated at room temperature with a blocking buffer (5% nonfat dry milk, 0.1% Triton-X100, 50 mM Tris, and 150mM NaCl). The membranes were then incubated overnight at 4°C with polyclonal antisera for G_{α11} (D-17; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), or G_{αq} (E-17; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), RGS7 (1:15,000 dilution), or RGS₄ (N-16 Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution). The 5-HT_{2A} receptor antibody (Singh, et al., 2007) was used at a dilution of 1:100,000 with overnight incubation. Next, the membranes were incubated at room temperature with a secondary antibody. For 5-HT_{2A} receptor, G_{α11}, G_{αq} and RGS7, we used peroxidase conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) at a 1:10,000-20,000 dilution. For RGS₄, we used rabbit anti-goat IgG (Sigma, St. Louis, MO) at a 1:2,000 dilution, and the blot was then incubated with goat peroxidase anti-peroxidase (ICN, Aurora, OH; 1:10,000 dilution). Membranes were incubated with monoclonal G_{β1-4} antibody (BD Biosciences Pharmingen, San Diego CA; 1:1,000) or monoclonal actin antibody (ICN, Aurora Ohio; 1:15,000) for 2 hours at room temperature followed by incubation in peroxidase conjugated goat-anti-mouse antibody at 1:5,000 for G_{β1-4} or 1:15,000 for actin. For Stat3, a polyclonal antibody from Cell Signaling (Beverly, MA) was used at 1:500 and for phospho-Stat3 (phosphorylated at Ser 727) a monoclonal antibody from Cell Signaling Technology (Danvers, MA) was used at 1:500. For both the Stat3 and phospho-Stat3, secondary antibody was used at 1:500 followed by peroxidase-anti-peroxidase

at 1:1,000. Actin or G β ₁₋₄ protein levels were measured on all blots and used to verify equal loading of protein in gels.

All nitrocellulose membranes were incubated with ECL enhanced chemiluminescence substrate solution (Amersham, Arlington Heights, IL) for 1 min and then exposed to Kodak x-ray film for 5 sec – 15 min. Films were analyzed densitometrically with the Scion Image program (Fredrick, MD). Gray scale density readings were calibrated using a transmission step wedge standard. The integrated optical densities (IOD) of each band were calculated within the linear range as the sum of the densities of all of the pixels within the area of the band outlined. An area adjacent to the band was used to calculate the background density of the band. The background IOD was subtracted from the IOD of each band. The data for each sample were the means of three replications. The mean IOD values of the treated samples were calculated with respect to the average of the IOD values of saline-treated samples. Western blot assays were repeated at least three times.

[¹²⁵I]-(±)-DOI Saturation Analysis

[¹²⁵I]-(±)-DOI with a specific activity of 2200 Ci/mmol was purchased from Perkin-Elmer Life Science, Inc. (Boston, MA). Frontal cortex from rats treated with either olanzapine 10mg/kg/day or vehicle for 7 days was homogenized using a Tekmar Tisumizer (Cincinnati, OH) in 30 volumes of ice-cold assay buffer containing 50mM Tris pH 7.4, 0.5 mM EDTA, 10 mM MgSO₄ (Battaglia, et al., 2000). The homogenate was centrifuged at 37,000× g for 15 minutes at 4°C. The resultant pellet was then resuspended again in the same buffer and centrifuged at 37,000× g for 15 minutes. The same process was repeated two more times, and a final membrane pellet was resuspended in assay buffer to reach a concentration of 20 mg wet weight/ml. The final amount of protein was determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

To determine changes in the density and affinity of high-affinity 5-HT_{2A} receptors increasing concentrations of [¹²⁵I]-(±)-DOI (0.4 – 4.0 nM) were incubated with the tissue homogenates (~50µg protein/tube) for 1.5 hour at room temperature in the same assay buffer as described above. The concentration range of [¹²⁵I]-(±)-DOI was based on the K_D in our previous study (Li, et al., 1997) and was over 7 times the K_D. This range of ligand (0.4 – 4.0 nM) resulted in up to 86% fractional occupancy of 5-HT_{2A} receptors. Non-specific binding was defined in the presence of 100 nM MDL 100,907, a selective 5-HT_{2A} receptor antagonist. The reaction was terminated by rapid filtration over Whatman GF/C glass fiber filters. The filters were then washed with 15 ml ice-cold 50 mM Tris buffer (pH 7.4). The amount of [¹²⁵I]-(±)-DOI on the filters was determined by using a Micromedic 4/200 Plus γ-counter. All radioligand receptor binding assays were performed in triplicate in a final volume of 0.5 ml. Specific binding was defined as the total binding minus nonspecific binding. B_{max} and K_D values determined by either computer-assisted analyses of saturation data using Prism (GraphPad Software, Inc., San Diego, CA) or by linear transformation of saturation data via Scatchard plots gave comparable results.

Statistics

For all statistical analyses, GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD) was used. All data are shown as a group mean ± SEM. PLC activity and 5-HT_{2A} receptor signaling system protein levels were analyzed using a one-way analysis of variance, followed by a Newman-Keuls' post hoc analysis. Stat3 and phospho-Stat3 levels in the rats treated with 10 mg/kg olanzapine and challenged with 1 mg/kg DOI were compared to saline-treated controls challenged with 1 mg/kg DOI using a Student's t-test.

Results

Body Weight

As shown in Figure 2, body weights of saline-treated rats increased over the treatment period. Unlike clinical studies with chronic olanzapine treatments in humans in which there is reported weight gain (Chrzanowski, et al., 2006), daily treatment with 0.5, 2 or 10 mg/kg olanzapine did not significantly alter weight gain over the 7 day treatment period.

PLC Activity in Frontal Cortex

Olanzapine treatment for 7 days caused a significant decrease in 5-HT-stimulated PLC activity in the rat frontal cortex ($F_{(3,20)} = 13.39$; $p < 0.0001$) as shown in Figure 3A. A post-hoc analysis revealed a decrease in activity by 53% with 0.5 mg/kg ($p < 0.01$), 58% with 2 mg/kg ($p < 0.01$), and 85% with 10 mg/kg treatment ($p < 0.01$) (Figure 3). As further shown in Figure 3B, treatment with olanzapine did not significantly affect GTP γ S-mediated PLC activity ($F_{(3,20)} = 1.01$; $p = 0.41$). These results suggest that the ability of the activated G proteins to stimulate PLC activity is not altered by chronic olanzapine.

[¹²⁵I]-($\pm$)-DOI binding in the Frontal Cortex

Treatment with 10mg/kg/day of olanzapine for 7 days produced a marked significant reduction in the high affinity state of 5-HT_{2A} receptors. The B_{max} of [¹²⁵I]-($\pm$)-DOI labeled 5-HT_{2A} receptors was decreased by 43% in the frontal cortex of olanzapine-treated rats compared to vehicle-treat controls (Student's t-test $t = -3.90$ with 4 degrees of freedom, $p < 0.05$). In addition a small but significant (34%) increase in the K_D for [¹²⁵I]-($\pm$)-DOI labeled 5-HT_{2A} receptors was also observed in frontal cortex of olanzapine-treated rats the (Table 1) as determine by a Student's t-test ($t = 6.30$ with 4 degrees of freedom, $p < 0.01$).

5-HT_{2A} Receptor Protein Levels in the Frontal Cortex

As shown in Figure 4A, membrane-associated 5-HT_{2A} receptor protein levels in the frontal cortex were dose-dependently increased by chronic treatment with olanzapine ($F_{(3,20)} = 3.86$, $p < 0.05$). Post-hoc analysis revealed that 5-HT_{2A} receptor proteins levels in rats treated with 10 mg/kg olanzapine were significantly different from saline-treated control rats. The levels of the 5-HT_{2A} receptor in rats treated with 10 mg/kg olanzapine were approximately twice the level of saline- treated control rats.

G Protein Levels in the Frontal Cortex

The levels of G β_{1-4} protein in the membrane-fraction of the frontal cortex of rats treated with olanzapine were not significantly different from saline-treated rats (Figure 4B). The levels of membrane-associated G α_q protein ($F_{(3,20)} = 13.20$, $p < 0.0001$) and G α_{11} protein ($F_{(3,22)} = 10.14$, $p < 0.001$) were significantly altered by olanzapine as shown in Figure 5A & B. There were dose-dependent increases in both G α_q and G α_{11} protein in the membrane fraction. In rats treated with 10 mg/kg olanzapine, the levels of both G α_q protein and G α_{11} protein in the membrane fraction were approximately double the level measured in saline-treated rats. G α_q protein and G α_{11} protein levels in the cytosolic fraction of the olanzapine-treated rats were not significantly different from the saline-treated control rats (Figure 5C & D).

RGS Protein Levels in the Frontal Cortex

Olanzapine significantly increased the levels of RGS4 protein in the membrane fraction of the frontal cortex ($F_{(3,23)} = 6.03$, $p < 0.01$) as shown in Figure 6A. These RGS4 protein levels were significantly higher in rats treated with 2 mg/kg (46% increased over controls) compared to saline-treated rats and rats treated with 0.5 mg/kg olanzapine (Figure 6A). RGS4 protein levels were also significantly higher in rats treated with 10 mg/kg of olanzapine (28% increased over

controls) compared to rats treated with 0.5 mg/kg olanzapine (Figure 6A). There were corresponding decreases (44%-43%) in the levels of RGS4 protein in the cytosolic fraction of rats treated with 2 and 10 mg/kg olanzapine ($F_{(3,20)} = 3.7$, $p < 0.05$, Figure 6B). The levels of RGS4 protein in the cytosol were also reduced in rats treated with 0.5 mg/kg olanzapine but there were no corresponding increases in the membrane-associated levels of RGS4 protein.

Analysis of variance revealed that RGS7 protein levels in the membrane-fraction were significantly increased ($F_{(3,22)} = 3.69$, $p < 0.05$) as shown in Figure 6C. The levels of RGS7 protein were also significantly increased in the cytosolic fraction of the frontal cortex of rats treated with olanzapine ($F_{(3,20)} = 14.5$, $p < 0.0001$; Figure 6D). Post-hoc analysis revealed that RGS7 protein levels in the cytosolic fraction from rats treated with 10 mg/kg olanzapine were statistically different from saline-treated control rats; the levels of RGS7 were more than twice the levels measured in saline-treated rats (Figure 6D).

Stat3 phosphorylation

In rats treated with 10 mg/kg olanzapine daily for 7 days and DOI-stimulated 30 minutes before sacrifice, Stat3 protein phosphorylation in the frontal cortex was significantly increased to over twice the levels in saline-treated rats ($t = -4.82$ with 10 degrees of freedom, $p < 0.001$, Figure 7). As shown in Figure 7, Stat3 protein levels were not altered in the frontal cortex by chronic treatment with 10 mg/kg olanzapine.

Discussion

In the present study, we found that olanzapine treatment reduced the function of the 5-HT_{2A} receptor signaling system, reduced the Bmax for [¹²⁵I]-(±)-DOI binding, altered expression of several proximate components of the system and increased activity of the Stat3 signal transduction pathway in rat frontal cortex. PLC activity was used as an index of the function (i.e., desensitization) of the 5-HT_{2A} receptor signaling system after chronic olanzapine treatment. Desensitization of 5-HT_{2A} receptor signaling caused by chronic treatment with olanzapine and other 5-HT_{2A} receptor antagonists is well established (for a review see (Gray and Roth, 2001)) and is repeated in the current study for direct comparison with the other measures such as protein expression and phosphorylation. Based on the half-life of olanzapine in rat brain of 2.5 to 5.1 hours (Aravagiri, et al., 1999; Kapur, et al., 2003) and the extensive washing of the membrane-fraction prior to the assay, olanzapine is not likely blocking 5-HT_{2A} receptors during the performance of the PLC assay and thus not likely directly impacting 5-HT-stimulated PLC activity measurements. We found that chronic olanzapine dose-dependently decreased 5-HT-stimulated PLC activity without significantly reducing GTPγS-stimulated PLC activity. These changes in agonist-stimulated PLC activity without an accompanying change in PLC activity with direct stimulation of G_{αq/11} proteins are similar to the effects produced by chronic agonist treatment (Damjanoska, et al., 2004). Our results using GTPγS to stimulate PLC activity suggest that the ability of activated G_{αq/11} proteins to stimulate second messengers is not altered by chronic olanzapine. In contrast, the ability of the agonist to stimulate the activation of PLC activity is reduced by olanzapine. Taken together, these results suggest that olanzapine alters 5-HT interaction with the 5-HT_{2A} receptors or decreases the ability of 5-HT_{2A} receptors to activate G_{αq/11} proteins.

A decrease in the ability of 5-HT_{2A} receptors to stimulate PLC is consistent with our results showing a decrease in the Bmax of [¹²⁵I] DOI labeled 5-HT_{2A} receptors in the frontal cortex of rats treated with 10mg/kg olanzapine for 7 days. It is also consistent with previous studies that showed that 5-HT_{2A} receptor antagonists reduce radiolabeled agonist binding to 5-HT_{2A} receptors (Kusumi, et al., 2000; Tarazi, et al., 2002) and that 5-HT_{2A} receptor antagonists induce internalization of 5-HT_{2A} receptors (Willins, et al., 1999). However, immunoblot analysis demonstrated that 5-HT_{2A} receptor protein levels in the membrane were increased rather than

decreased by olanzapine treatment. Olanzapine exposure also increased the 5-HT_{2A} receptor protein levels measured by immunoblot analysis, in A1A1v cells in culture (Singh, et al., 2007). There are at least two possible explanations for the differences between our results with immunoblot analysis of 5-HT_{2A} receptors and our 5-HT_{2A} receptor binding results as well as previous findings demonstrating decreased receptor binding and internalization. First, olanzapine may alter the ability of the 5-HT_{2A} receptor protein to bind agonist or second, to bind G proteins possibly through alterations in post-translational modifications such as phosphorylation. In this scenario, total 5-HT_{2A} receptor protein levels could increase despite a decrease in agonist binding if the post-translational modification directly reduced the number of receptors that can bind to the agonist or indirectly altered agonist binding by interfering with the ability of the receptor to bind to G α proteins, and thus maintaining the receptors in a low affinity state. Phosphorylation of 5-HT_{2A} receptors has been described in cells in culture (Gray, et al., 2003) and we have demonstrated phosphorylation of G α_{11} protein that causes desensitization of 5-HT_{2A} receptor signaling (Shi, et al., 2007). Considering that the 5-HT_{2A} receptor antibody epitope is based on the primary amino acid sequence, it is feasible to obtain differences in levels of 5-HT_{2A} receptors detected by immunoblot analysis versus changes detected by radioligand binding studies.

Additionally, we found an increase in membrane-associated RGS proteins following olanzapine treatment. An increase in membrane-associated RGS proteins 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 GTP γ S, a non-hydrolysable GTP analog is used to activate G proteins as in our PLC assay, RGS proteins are not able to hydrolyze GTP γ S 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 RGS protein as an underlying mechanism for olanzapine-induced desensitization.

Both RGS4 and RGS7 protein levels were increased in the membrane fraction following chronic treatment with olanzapine. RGS4 protein levels in the membrane were increased by an amount similar to the decrease in the cytosol with 2.0 and 10 mg/kg olanzapine treatments suggesting a redistribution of RGS4 proteins. Previous *in vivo* and *in vitro* studies demonstrated that RGS4 protein function is dependent on membrane association (Tu, et al., 2001). The redistribution of RGS4 protein following chronic olanzapine treatment would thereby increase the rate of hydrolysis of activated G $\alpha_{q/11}$ and could contribute to the desensitization response. The mechanism whereby chronic olanzapine causes a redistribution of RGS4 protein is not clear but could be due to the increased expression of G $\alpha_{q/11}$ protein and 5-HT_{2A} receptor protein. A previous study demonstrated that localization of RGS protein to the membrane increases with increased expression of G α protein (Roy et al, 2003). However, in the cell culture system used in that study, G α_i protein but not G α_q protein expression increased association of RGS4 with the cell membrane. Increased G α_o but not G α_q protein expression increased palmitoylation and membrane association of RGS7 (Takida, et al., 2005). G α_{11} was not examined in that study but could also impact the distribution of RGS protein. Increased expression of receptor proteins also increases membrane association of RGS protein (Roy, et al., 2003) suggesting that increased expression of 5-HT_{2A} receptors could also increase RGS4 protein association with the cell membrane.

RGS7 protein levels increased in both the membrane and cytosol with 10 mg/kg olanzapine treatment. Expression of RGS4 and RGS7 have been previously noted to be independent (Krumins, et al., 2004). RGS7 phosphorylation and subsequent binding to 14-3-3 sequesters RGS7 in the cytoplasm (Burchett, 2003). Furthermore, RGS7 binding to G β_5 is necessary for stability of each protein (Chen, et al., 2003). Therefore, an increase in phosphorylation of RGS7 or increased expression of 14-3-3 or G β_5 could increase the levels of RGS7 in the cytoplasm. RGS4 does not bind to 14-3-3 or G β_5 as RGS7 does. In contrast with olanzapine-treatment, a

similar treatment schedule with the 5-HT_{2A} receptor agonist DOI produces 5-HT_{2A} receptor desensitization without changing RGS7 protein expression in the frontal cortex (Damjanoska et al., 2004) possibly suggesting different underlying mechanisms for induction of desensitization with agonists versus antagonists. Our observed increase in membrane-associated RGS4 and RGS7 could both contribute to the desensitization response caused by olanzapine.

While the membrane-associated component of G_{αq} and G_{α11} proteins increased with 10 mg/kg olanzapine treatments, the cytosolic levels of these proteins were unchanged. Our previous study in transgenic rats over-expressing G_{αq} protein showed a similar increase in only the membrane-associated fraction of G_{αq} protein and no change in the cytosolic fraction (Shi, et al., 2006). These results suggest that the membrane and cytosolic fractions are not in an equilibrium relationship since this would predict that both the membrane and cytosolic components would increase with an increase in expression of G_{αq/11} proteins. The constant levels of G_{αq/11} protein in the cytosol in these models suggest that the levels of G_{αq/11} in the cytosol are statically regulated while membrane-associated G_{αq/11} proteins are dynamic and follow expression levels of the proteins. An increase in G_{αq/11} proteins would be expected when an increase rather than a decrease in 5-HT_{2A} receptor signaling is detected and therefore the increase in membrane-associated G_{αq/11} proteins could not contribute to the desensitization response caused by chronic olanzapine. Other studies in our laboratory suggest that phosphorylation of G_{α11} protein contributes to desensitization of 5-HT_{2A} receptor signaling induced by DOI treatment (Shi, et al., 2007) and could mitigate the effects of increased levels of G_α proteins. We are currently determining whether phosphorylation of G_{α11} protein occurs with chronic olanzapine treatment as well.

Not all of the G proteins examined after olanzapine treatment showed an increased expression. G_{β1-4} protein levels in the membrane were not altered by chronic olanzapine treatment, demonstrating that chronic olanzapine produces selective increases in proteins associated with 5-HT_{2A} receptor signaling.

One mechanism by which olanzapine could alter the expression levels of RGS proteins and other signaling proteins is via the increase in Stat3 activity that occurred in the frontal cortex following chronic treatment with olanzapine. We previously demonstrated in A1A1v cells, that olanzapine also increased Stat3 phosphorylation and caused a Jak2/Stat3 dependent-increase in membrane-associated RGS7 protein (Singh, et al., 2007). The Jak/Stat pathway increases immediate-early gene expression including expression of c-Fos, c-Jun and c-Myc (Burysek, et al., 2002; Cattaneo, et al., 1999). Indeed, olanzapine has been shown to increase the number of Fos-positive cells in rat frontal cortex (Sebens, et al., 1998). These transcription factors could subsequently increase expression of select genes such as those encoding G_{αq}, G_{α11}, and RGS7 proteins found to be increased in this study after chronic olanzapine treatment. The increase in 5-HT_{2A} receptor protein levels is not dependent on Jak2/Stat3 signaling as RGS7 is in A1A1v cells (Singh et al., 2007) and may be regulated similarly *in vivo*.

In conclusion, chronic olanzapine caused a dose-dependent desensitization of 5-HT_{2A} receptor signaling, a decrease in the Bmax for [¹²⁵I]-(±)-DOI binding and an increase in the expression of several proteins in the 5-HT_{2A} receptor signaling pathway in the membrane fraction of rat frontal cortex. Olanzapine caused an increase in Stat3 phosphorylation and thus an increase in activity of Stat3 that could underlie the increase in expression of these proteins in the 5-HT_{2A} receptor pathway. Notably, the increase in RGS4 and RGS7 proteins in the membrane could contribute to the desensitization response induced by chronic olanzapine treatment. The time course for the full therapeutic effects of olanzapine in humans is 2-3 weeks while the current study examined the effects of olanzapine after only 1 week of daily treatment. Future

studies are needed to determine if the changes in protein expression especially the RGS proteins are maintained with longer treatment regimens.

Acknowledgments

The authors are grateful to Dr. Susan Luecke at Eli Lilly and Company for the generous donation of olanzapine and Drs. Philip Jones and Kathleen Young (Wyeth-Ayerst Research, Princeton, N.J., U.S.A.) for their generous donation of rabbit polyclonal RGS7 antibody.

Supported by USPHS MH068612 and NS034153.

References

- Anji A, Kumari M, Hanley NRS, Bryan GL, Hensler JG. Regulation of 5-HT_{2A} receptor mRNA levels and binding sites in rat frontal cortex by the agonist DOI and the antagonist mianserin. *Neuropharmacology* 2000;39:1996–2005. [PubMed: 10963743]
- Aravagiri M, Teper Y, Marder SR. Pharmacokinetics and tissue distribution of olanzapine in rats. *Biopharm Drug Dispos* 1999;20:369–377. [PubMed: 10870093]
- Battaglia G, Cabrera-Vera TM, Van de Kar LD. Prenatal cocaine exposure potentiates 5-HT_{2A} receptor function in male and female rat offspring. *Synapse* 2000;35:163–172. [PubMed: 10657023]
- Bhatnagar A, Willins DL, Gray JA, Woods J, Benovic JL, Roth BL. The dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT_{2A}) serotonin receptors reveals differential sorting of arrestins and 5-HT_{2A} receptors during endocytosis. *J Biol Chem* 2001;276:8269–8277. [PubMed: 11069907]
- Burchett SA. In through the out door: nuclear localization of the regulators of G protein signaling. *J Neurochem* 2003;87:551–559. [PubMed: 14535939]
- Burysek L, Syrovets T, Simmet T. The Serine Protease Plasmin Triggers Expression of MCP-1 and CD40 in Human Primary Monocytes via Activation of p38 MAPK and Janus Kinase (JAK)/STAT Signaling Pathways. *J Biol Chem* 2002;277:33509–33517. [PubMed: 12093796]
- Cattaneo E, Conti L, De-Fraja C. Signalling through the JAK-STAT pathway in the developing brain. *Trends Neurosci* 1999;22:365–369. [PubMed: 10407422]
- Chen CK, Eversole-Cire P, Zhang H, Mancino V, Chen YJ, He W, Wensel TG, Simon MI. Instability of GGL domain-containing RGS proteins in mice lacking the G protein beta-subunit Gbeta5. *Proc Natl Acad Sci U S A* 2003;100:6604–6609. [PubMed: 12738888]
- Chrzanowski WK, Marcus RN, Torbeyns A, Nyilas M, McQuade RD. Effectiveness of long-term aripiprazole therapy in patients with acutely relapsing or chronic, stable schizophrenia: a 52-week, open-label comparison with olanzapine. *Psychopharmacology (Berl)* 2006;189:259–266. [PubMed: 17058105]
- Corya SA, Williamson D, Sanger TM, Briggs SD, Case M, Tollefson G. A randomized, double-blind comparison of olanzapine/fluoxetine combination, olanzapine, fluoxetine, and venlafaxine in treatment-resistant depression. *Depress Anxiety*. 2006
- Damjanoska KJ, Heidenreich BA, Kindel GH, D'Souza DN, Zhang Y, Garcia F, Battaglia G, Wolf WA, Van de Kar LD, Muma NA. Agonist-Induced Serotonin 2A Receptor Desensitization in the Rat Frontal Cortex and Hypothalamus. *J Pharmacol Exp Ther* 2004;309:1043–1050. [PubMed: 14976228]
- Damjanoska KJ, Van de Kar LD, Kindel GH, Zhang Y, D'Souza DN, Garcia F, Battaglia G, Muma NA. Chronic fluoxetine differentially affects 5-HT_{2A} receptor signaling in frontal cortex, oxytocin and corticotropin releasing factor (CRF)-containing neurons in rat paraventricular nucleus. *J Pharmacol Exp Ther* 2003;306:563–571. [PubMed: 12721328]
- Ghavami A, Hunt RA, Olsen MA, Zhang J, Smith DL, Kalgaonkar S, Rahman Z, Young KH. Differential effects of regulator of G protein signaling (RGS) proteins on serotonin 5-HT_{1A}, 5-HT_{2A}, and dopamine D₂ receptor-mediated signaling and adenylyl cyclase activity. *Cell Signal* 2004;16:711–721. [PubMed: 15093612]
- Gray JA, Compton-Toth BA, Roth BL. Identification of two serine residues essential for agonist-induced 5-HT_{2A} receptor desensitization. *Biochemistry* 2003;42:10853–10862. [PubMed: 12962510]

- Gray JA, Roth BL. Paradoxical trafficking and regulation of 5HT_{2A} receptors by agonists and antagonists. *Brain Research Bulletin* 2001;56:441–451. [PubMed: 11750789]
- Guillet-Deniau I, Burnol AF, Girard J. Identification and localization of a skeletal muscle serotonin 5-HT_{2A} receptor coupled to the Jak/STAT pathway. *J Biol Chem* 1997;272:14825–14829. [PubMed: 9169451]
- Kapur S, VanderSpek SC, Brownlee BA, Nobrega JN. Antipsychotic dosing in preclinical models is often unrepresentative of the clinical condition: a suggested solution based on in vivo occupancy. *J Pharmacol Exp Ther* 2003;305:625–631. [PubMed: 12606608]
- Krumins AM, Barker SA, Huang C, Sunahara RK, Yu K, Wilkie TM, Gold SJ, Mumby SM. Differentially regulated expression of endogenous RGS4 and RGS7. *J Biol Chem* 2004;279:2593–2599. [PubMed: 14604980]
- Kusumi I, Takahashi Y, Suzuki K, Kameda K, Koyama T. Differential effects of subchronic treatments with atypical antipsychotic drugs on dopamine D2 and serotonin 5-HT_{2A} receptors in the rat brain. *J Neural Transm* 2000;107:295–302. [PubMed: 10821438]
- Li Q, Muma NA, Battaglia G, Van de Kar LD. Fluoxetine gradually increases [¹²⁵I]DOI-labelled 5-HT_{2A/2C} receptors in the hypothalamus without changing the levels of G_q- and G₁₁-proteins. *Brain Res* 1997;775:225–228. [PubMed: 9439849]
- Marek GJ, Carpenter LL, McDougle CJ, Price LH. Synergistic action of 5-HT_{2A} antagonists and selective serotonin reuptake inhibitors in neuropsychiatric disorders. *Neuropsychopharmacology* 2003;28:402–412. [PubMed: 12589395]
- Pierce KL, Lefkowitz RJ. Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci* 2001;2:727–733. [PubMed: 11584310]
- Ram PT, Iyengar R. G protein coupled receptor signaling through the Src and Stat3 pathway: role in proliferation and transformation. *Oncogene* 2001;20:1601–1606. [PubMed: 11313907]
- Roth BL, Willins DL, Kristiansen K, Kroeze WK. 5-Hydroxytryptamine₂-family (hydroxytryptamine_{2A}, hydroxytryptamine_{2B}, hydroxytryptamine_{2C}): where structure meets function. *Pharmacol Ther* 1998;79:231–257. [PubMed: 9776378]
- Roy AA, Lemberg KE, Chidiac P. Recruitment of RGS2 and RGS4 to the plasma membrane by G proteins and receptors reflects functional interactions. *Mol Pharmacol* 2003;64:587–593. [PubMed: 12920194]
- Sebens JB, Koch T, ter Horst GJ, Korf J. Olanzapine-induced Fos expression in the rat forebrain; cross-tolerance with haloperidol and clozapine. *Eur J Pharmacol* 1998;353:13–21. [PubMed: 9721035]
- Shi J, Damjanoska KJ, Zemaitaitis BW, Garcia F, Carrasco GA, Sullivan NR, She Y, Young KH, Battaglia G, Van de Kar LD, Howland DS, Muma NA. Alterations in 5-HT_{2A} receptor signaling in male and female transgenic rats overexpressing either G_q or RGS-insensitive G_q protein. *Neuropharmacology* 2006;51:524–536. [PubMed: 16769091]
- Shi J, Zemaitaitis B, Muma NA. Phosphorylation of Gα₁₁ protein contributes to agonist-induced desensitization of 5-HT_{2A} receptor signaling. *Mol Pharmacol* 2007;71:303–313. [PubMed: 17056873]
- Shuey DJ, Betty M, Jones PG, Khawaja XZ, Cockett MI. RGS7 attenuates signal transduction through the G_{αq} family of heterotrimeric G proteins in mammalian cells. *J Neurochem* 1998;70:1964–1972. [PubMed: 9572280]
- Singh RK, Shi J, Zemaitaitis B, Muma NA. Olanzapine increases RGS7 protein expression via increased activation of the JAK/Stat signaling cascade. *J Pharmacol Exp Ther*. 2007In press
- Takida S, Fischer CC, Wedegaertner PB. Palmitoylation and plasma membrane targeting of RGS7 are promoted by alpha o. *Mol Pharmacol* 2005;67:132–139. [PubMed: 15496508]
- Tarazi FI, Zhang KH, Baldessarini RJ. Long-term effects of olanzapine, risperidone, and quetiapine on serotonin 1A, 2A and 2C receptors in rat forebrain regions. *Psychopharmacology* 2002;161:263–270. [PubMed: 12021829]
- Tilakaratne N, Yang ZL, Friedman E. Chronic fluoxetine or desmethylimipramine treatment alters 5-HT₂ receptor mediated c-fos gene expression. *Eur J Pharmacol Mol Pharmacol* 1995;290:263–266.
- Tohen M, Vieta E, Calabrese J, Ketter TA, Sachs G, Bowden C, Mitchell PB, Centorrino F, Risser R, Baker RW, Evans AR, Beymer K, Dube S, Tollefson GD, Breier A. Efficacy of olanzapine and

olanzapine-fluoxetine combination in the treatment of bipolar I depression. Arch Gen Psychiatry 2003;60:1079–1088. [PubMed: 14609883]

Tu YP, Woodson J, Ross EM. Binding of regulator of G protein signaling (RGS) proteins to phospholipid bilayers - Contribution of location and/or orientation to GTPase-activating protein activity. J Biol Chem 2001;276:20160–20166. [PubMed: 11274219]

Willins DL, Berry SA, Alsayegh L, Backstrom JR, Sanders-Bush E, Friedman L, Roth BL. Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors *in vitro* and *in vivo*. Neuroscience 1999;91:599–606. [PubMed: 10366017]

Wolf WA, Schutz LJ. The 5-HT_{2C} receptor is a prominent 5-HT receptor in basal ganglia: evidence from functional studies on 5-HT-mediated phosphoinositide hydrolysis. J Neurochem 1997;69:1449–1458. [PubMed: 9326273]

Hypothetical 5-HT_{2A} receptor signal transduction pathways

Figure 1.

5-HT_{2A} receptor agonists cause activation of G_{αq/11} proteins which in turn activate the second messenger enzyme PLC. Chronic treatment with olanzapine causes desensitization of this pathway as measured by either production of inositol phosphate in cells or PLC activity in brain tissue. We hypothesize that 5-HT_{2A} receptor antagonism (induced by olanzapine) activates the Jak2/Stat3 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. The increased membrane-associated RGS7 protein can then increase hydrolysis of activated G_{αq/11} and result in desensitization of 5-HT_{2A} receptor signaling.

Figure 2.

Rat body weight is not altered by 0.5, 2, or 10 mg/kg olanzapine injected daily for 1, 3, 5, or 7 days.

Figure 3.

(A) 5-HT-stimulated PLC activity in the frontal cortex was significantly attenuated by daily treatments with olanzapine for 7 days. Each dose of olanzapine (Olan), 0.5, 2.0 and 10.0 mg/kg, significantly reduced PLC activity compared to saline-treated controls (** indicates significantly different from saline-treated controls rats at $p < 0.01$). (B) GTP γ S-stimulated-PLC activity was not altered by the olanzapine treatments.

Figure 4.

(A) Olanzapine (Olan) treatment increased the membrane-associated levels of the 5-HT_{2A} receptor protein in rat frontal cortex measured on western blots. (B) There were no changes in the levels of G β ₁₋₄ in the membrane fraction with olanzapine treatment. Actin was used to verify equal loading of lanes in the SDS PAGE gel. * indicates significantly different from saline-treated controls at $p < 0.05$.

Membrane-associated $G\alpha$ Cytosol-associated $G\alpha$ **Figure 5.**

Chronic olanzapine differentially altered $G\alpha$ protein levels in the cytosolic and membrane fraction of the frontal cortex as measured by western blotting. The levels of $G_{\alpha q}$ (A) and $G_{\alpha 11}$ (B) in the membrane fraction were significantly increased in the rats treated with 10 mg/kg olanzapine. In contrast, the levels of $G_{\alpha q}$ (C) and $G_{\alpha 11}$ (D) proteins in the cytosol fraction of rat frontal cortex were not significantly altered by chronic olanzapine treatments. * indicates significantly different from saline-treated control rats at $p < 0.05$.

Membrane-associated RGS Protein

Cytosol-associated RGS Protein

Figure 6.

Chronic olanzapine differentially altered RGS4 and RGS7 protein levels in the frontal cortex. RGS4 levels in the membrane fraction (A) were increased by daily treatment with 2.0 and 10.0 mg/kg olanzapine. The levels of RGS4 in the cytosol (B) were reduced by approximately half with all doses of olanzapine. In contrast, RGS7 protein levels in the membrane (C) and cytosol (D) were significantly increased by daily injections of 10 mg/kg olanzapine. * indicates significantly different from saline-treated control rats and # indicates significantly different compared to 0.5 mg/kg olanzapine at $p < 0.05$.

Figure 7.

Chronic olanzapine increased Stat3 activity in the frontal cortex. The levels of phosphorylated Stat 3 were significantly increased by daily treatments of 10 mg/kg olanzapine (Olan 10). Stat protein levels were not altered by olanzapine. Actin protein levels were used to verify equal loading of lanes on the SDS-PAGE gel. * indicates significantly different from saline-treated control rats at $p < 0.05$.

Table 1The effects of olanzapine on [125 I]-($\pm$)-DOI binding in rat frontal cortex

	K_D	B_{max}
Control	0.56 $\pm$ 0.04 (mean $\pm$ SEM)	48.0 $\pm$ 8.6 (mean $\pm$ SEM)
Olanzapine	0.75 $\pm$ 0.03 (mean $\pm$ SEM)	27.4 $\pm$ 3.1 (mean $\pm$ SEM)
Percent change	34% increase	43% decrease