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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☆

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Abstract

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, $G_{\alpha 11}$ and $G_{\alpha q}$ 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 $G_{\alpha q/11}$ proteins more rapidly.

Keywords: Antipsychotic; Serotonin 2A receptor; In vivo

1. Introduction

Adaptive changes in post-synaptic serotonin2A/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 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

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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 $G_{\alpha q/11}$ proteins to increase the activity of phospholipase C (PLC) (Roth et al., 1998). Hydrolysis and thereby termination of 5-HT_{2A} receptor-activated $G_{\alpha q/11}$ 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 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. 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-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 (Singh et al., 2007). Based on these previous studies in cell culture and as shown in Fig. 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.

2. Methods

2.1. 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 per 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-lodophenyl)-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

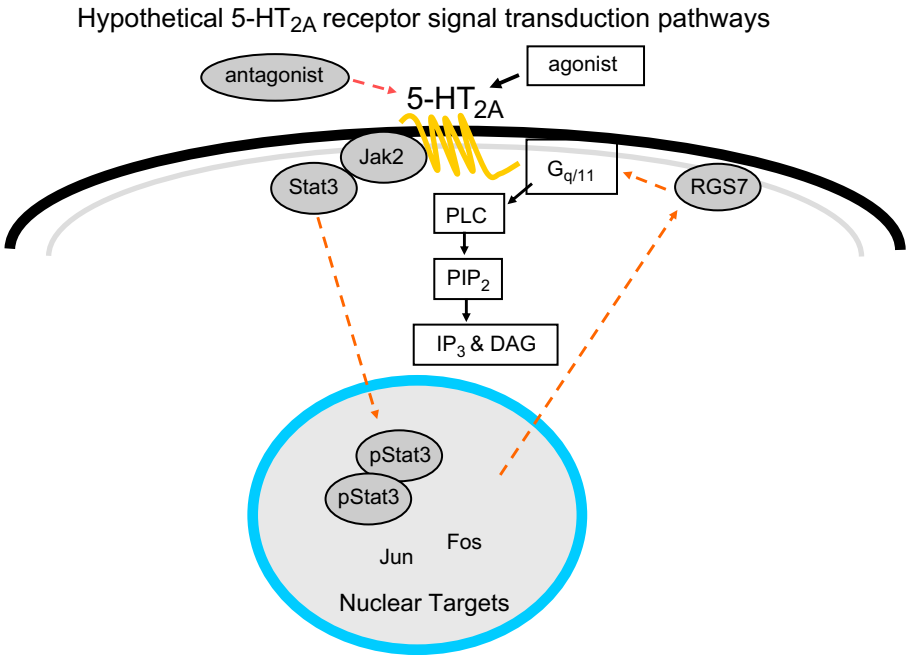

Fig. 1. 5-HT_{2A} receptor agonists cause activation of $G_{\alpha q/11}$ proteins which in turn activate the second messenger enzyme PLC. Chronic treatment with olanzapine causes desensitization of this pathway as measured by either production of inositol phosphate in cells or PLC activity in brain 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 $G_{\alpha q/11}$ and result in desensitization of 5-HT_{2A} receptor signaling.

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

2.2. 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 (1.5 μL/30 mg tissue; St. Louis, MO). Phosphatase inhibitors (50 mM NaF and 1 mM Na_3VO_4) were also added to the homogenation buffer. The protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

2.3. 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.

2.4. Immunoblots

Immunoblot analysis of proteins has been described previously in detail (Damjanoska et al., 2003). Briefly, the membrane or cytosolic proteins (10–2.5 μg/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% non-fat dry milk, 0.1% Triton-X100, 50 mM Tris, and 150 mM NaCl). The membranes were then incubated overnight at 4 °C with polyclonal antisera for $G_{\alpha 11}$ (D-17; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), or $G_{\alpha q}$ (E-17; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), RGS7 (1:15,000 dilution), or RGS_4 (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, $G_{\alpha 11}$, $G_{\alpha q}$ 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 1:2000 dilution, and the blot was then incubated with goat peroxidase anti-peroxidase (ICN, Aurora, OH; 1:10,000 dilution). Membranes were incubated with monoclonal $G_{\beta 1-4}$ antibody (BD Biosciences Pharmingen, San Diego CA; 1:1000) or monoclonal actin antibody (ICN, Aurora Ohio; 1:15,000) for 2 h at room temperature followed by incubation in peroxidase conjugated goat-anti-mouse antibody at 1:5000 for $G_{\beta 1-4}$ 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 1:1000. Actin or $G_{\beta 1-4}$ 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 s–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.

2.5. [^{125}I]-(±)-DOI saturation analysis

[^{125}I]-(±)-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 10 mg/kg per day or vehicle for 7 days was homogenized using a Tekmar Tissumizer (Cincinnati, OH) in 30 volumes of ice-cold assay buffer containing 50 mM Tris pH 7.4, 0.5 mM EDTA, 10 mM $MgSO_4$ (Battaglia et al., 2000). The homogenate was centrifuged at 37,000 × *g* for 15 min at 4 °C. The resultant pellet was then resuspended again in the same buffer and centrifuged at 37,000 × *g* for 15 min. 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 [^{125}I]-(±)-DOI (0.4–4.0 nM) were incubated with the tissue homogenates (~50 μg protein/tube) for 1.5 h at room temperature in the same assay buffer as described above. The concentration range of [^{125}I]-(±)-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 [^{125}I]-(±)-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 non-specific binding. Bmax 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.

2.6. 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.

3. Results

3.1. Body weight

As shown in Fig. 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.

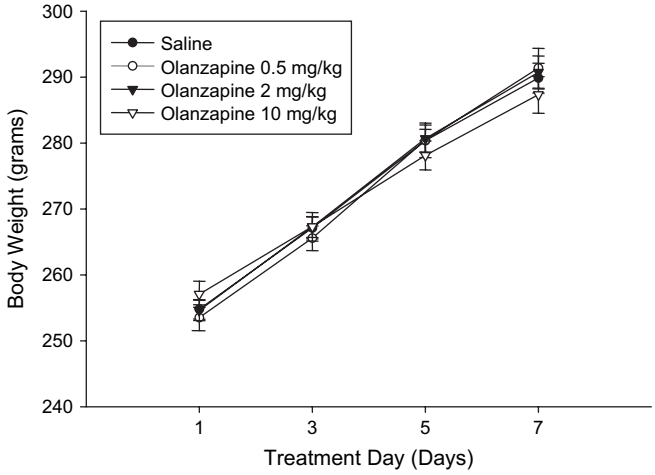

Fig. 2. Rat body weight is not altered by 0.5, 2, or 10 mg/kg olanzapine injected daily for 1, 3, 5, or 7 days.

3.2. 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 Fig. 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 ($p < 0.01$) (Fig. 3). As further shown in Fig. 3B, treatment with olanzapine did not significantly affect GTPγS-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.

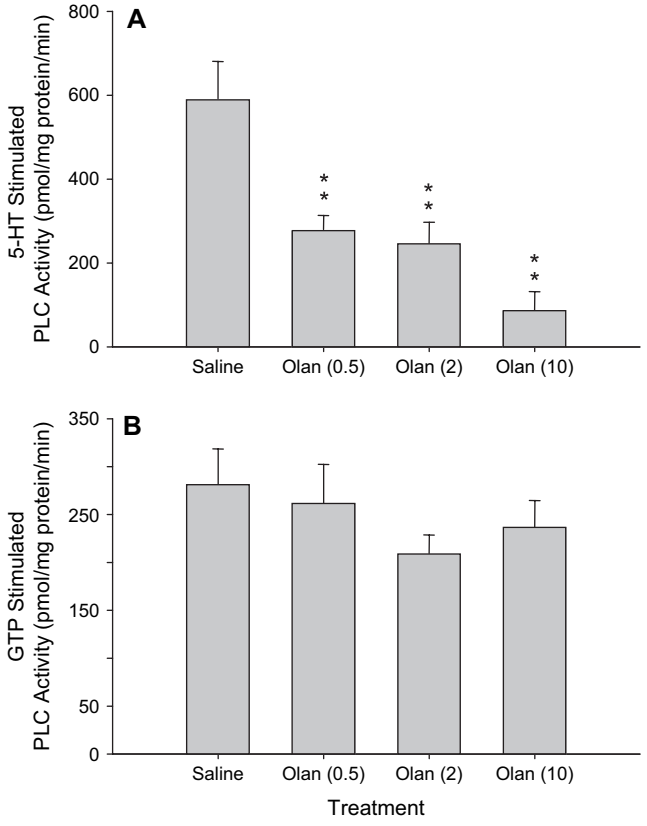

Fig. 3. (A) 5-HT-stimulated PLC activity in the frontal cortex was significantly attenuated by daily treatments with olanzapine for 7 days. Each dose of olanzapine (Olan), 0.5, 2.0 and 10.0 mg/kg, significantly reduced PLC activity compared to saline-treated controls (**indicates significantly different from saline-treated controls rats at $p < 0.01$). (B) GTPγS-stimulated-PLC activity was not altered by the olanzapine treatments.

3.3. [^{125}I]-(±)-DOI binding in the frontal cortex

Treatment with 10 mg/kg per day of olanzapine for 7 days produced a marked significant reduction in the high affinity state of 5-HT$_{2A}$ receptors. The Bmax of [^{125}I]-(±)-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 [^{125}I]-(±)-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, $p < 0.01$).

3.4. 5-HT$_{2A}$ receptor protein levels in the frontal cortex

As shown in Fig. 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.

3.5. G protein levels in the frontal cortex

The levels of $G_{\beta 1-4}$ protein in the membrane-fraction of the frontal cortex of rats treated with olanzapine were not significantly different from saline-treated rats (Fig. 4B). The levels of membrane-associated $G_{\alpha q}$ protein ($F_{(3,20)} = 13.20$, $p < 0.0001$) and $G_{\alpha 11}$ protein ($F_{(3,22)} = 10.14$, $p < 0.001$) were significantly altered by olanzapine as shown in Figs. 5A,B. There were dose-dependent increases in both $G_{\alpha q}$ and $G_{\alpha 11}$ protein in the membrane fraction. In rats treated with 10 mg/kg olanzapine, the levels of both $G_{\alpha q}$ protein and $G_{\alpha 11}$ protein in the membrane fraction were approximately double the level measured in saline-treated rats. $G_{\alpha q}$ protein and

Table 1
The effects of olanzapine on [^{125}I]-(±)-DOI binding in rat frontal cortex

	K_D	Bmax
Control	0.56 ± 0.04 (mean ± SEM)	48.0 ± 8.6 (mean ± SEM)
Olanzapine	0.75 ± 0.03 (mean ± SEM)	27.4 ± 3.1 (mean ± SEM)
Percent change	34% increase	43% decrease

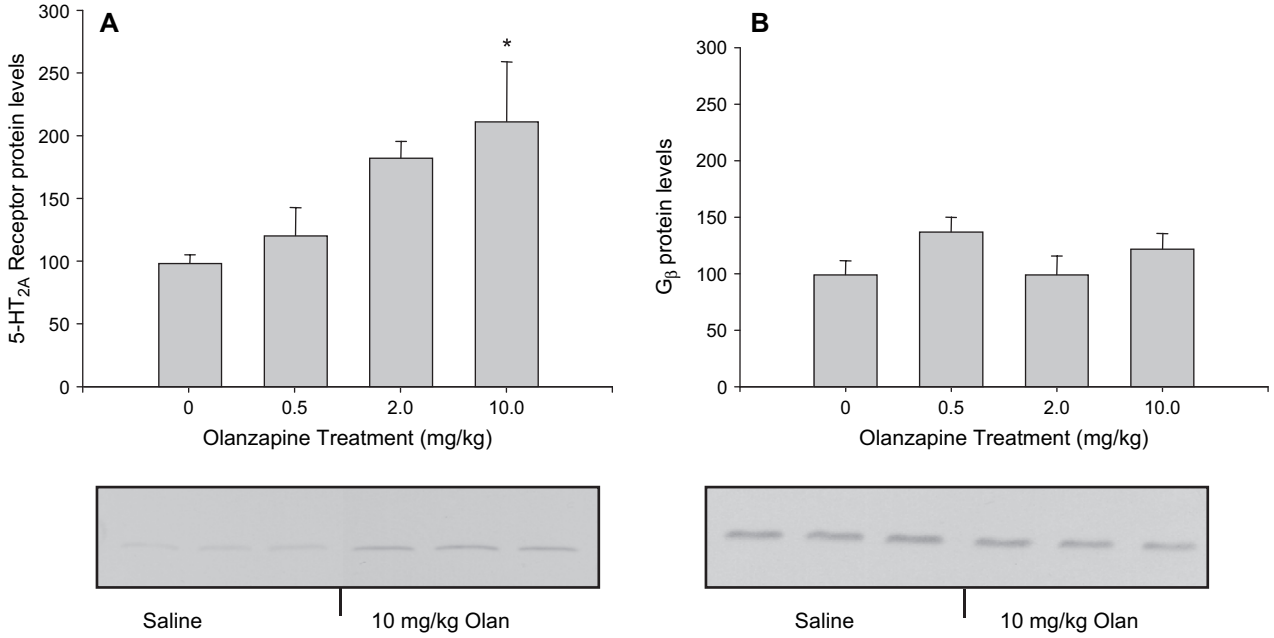

Fig. 4. (A) Olanzapine (Olan) treatment increased the membrane-associated levels of the 5-HT_{2A} receptor protein in rat frontal cortex measured on western blots. (B) There were no changes in the levels of $G_{\beta 1-4}$ in the membrane fraction with olanzapine treatment. Actin was used to verify equal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

$G_{\alpha 11}$ protein levels in the cytosolic fraction of the olanzapine-treated rats were not significantly different from the saline-treated control rats (Figs. 5C,D).

3.6. 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 Fig. 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 (Fig. 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 (Fig. 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 ($F_{(3,20)} = 3.7$, $p < 0.05$, Fig. 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 Fig. 6C. The levels of RGS7 protein were also significantly increased in the cytosolic fraction of the frontal cortex of rats treated with olanzapine ($F_{(3,20)} = 14.5$, $p < 0.0001$; Fig. 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 (Fig. 6D).

3.7. Stat3 phosphorylation

In rats treated with 10 mg/kg olanzapine daily for 7 days and DOI-stimulated 30 min 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, $p < 0.001$, Fig. 7). As shown in Fig. 7, Stat3 protein levels were not altered in the frontal cortex by chronic treatment with 10 mg/kg olanzapine.

4. 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 [^{125}I]-(±)-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 2.5–5.1 h (Aravagiri et al., 1999; Kapur et al., 2003) and

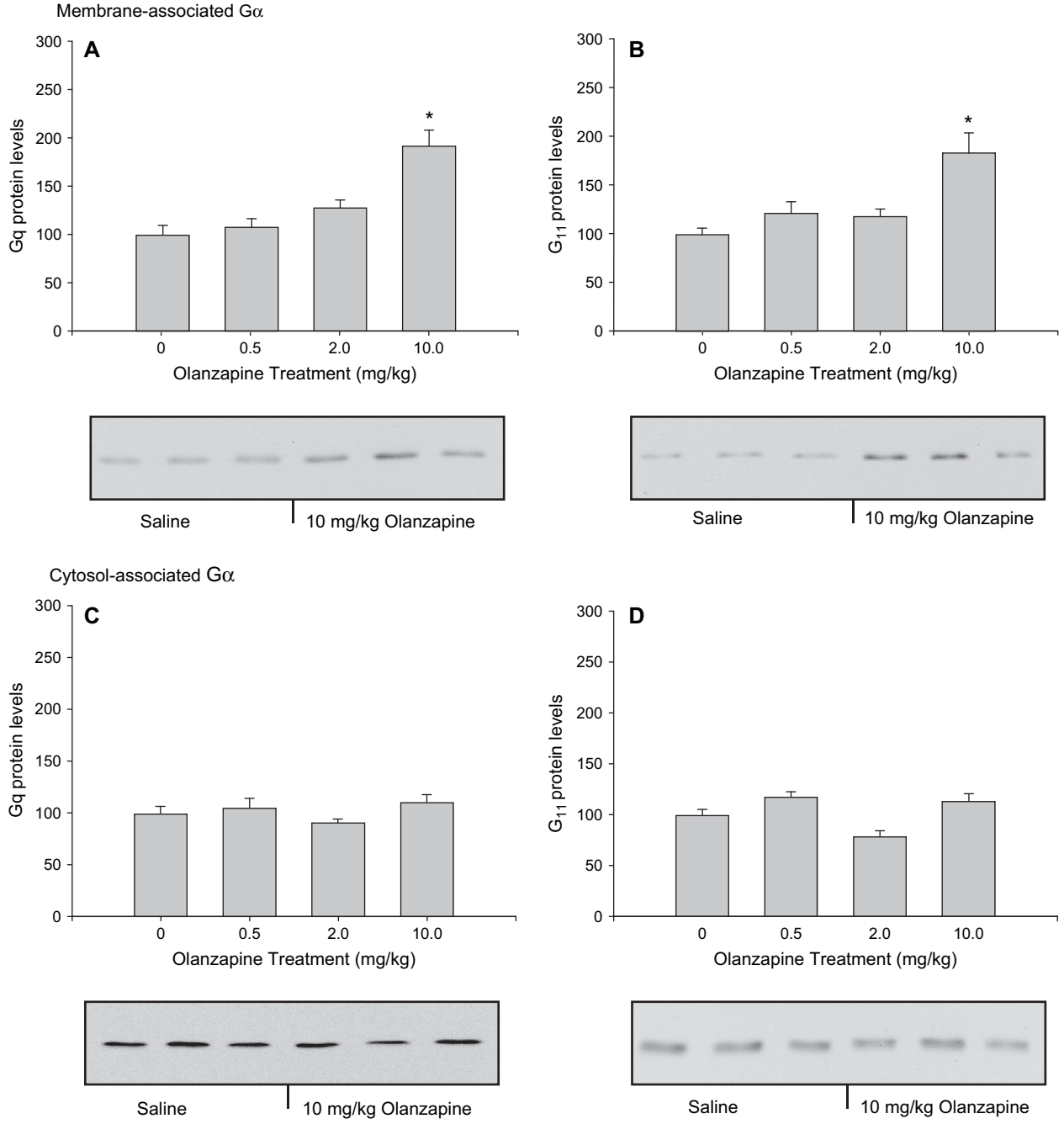

Fig. 5. Chronic olanzapine differentially altered G_{α} protein levels in the cytosolic and membrane fraction of the frontal cortex as measured by western blotting. The levels of $G_{\alpha q}$ (A) and $G_{\alpha 11}$ (B) in the membrane fraction were significantly increased in the rats treated with 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 $p < 0.05$.

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 $G_{\alpha q/11}$ 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 $G_{\alpha q/11}$ 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 $G_{\alpha q/11}$ proteins.

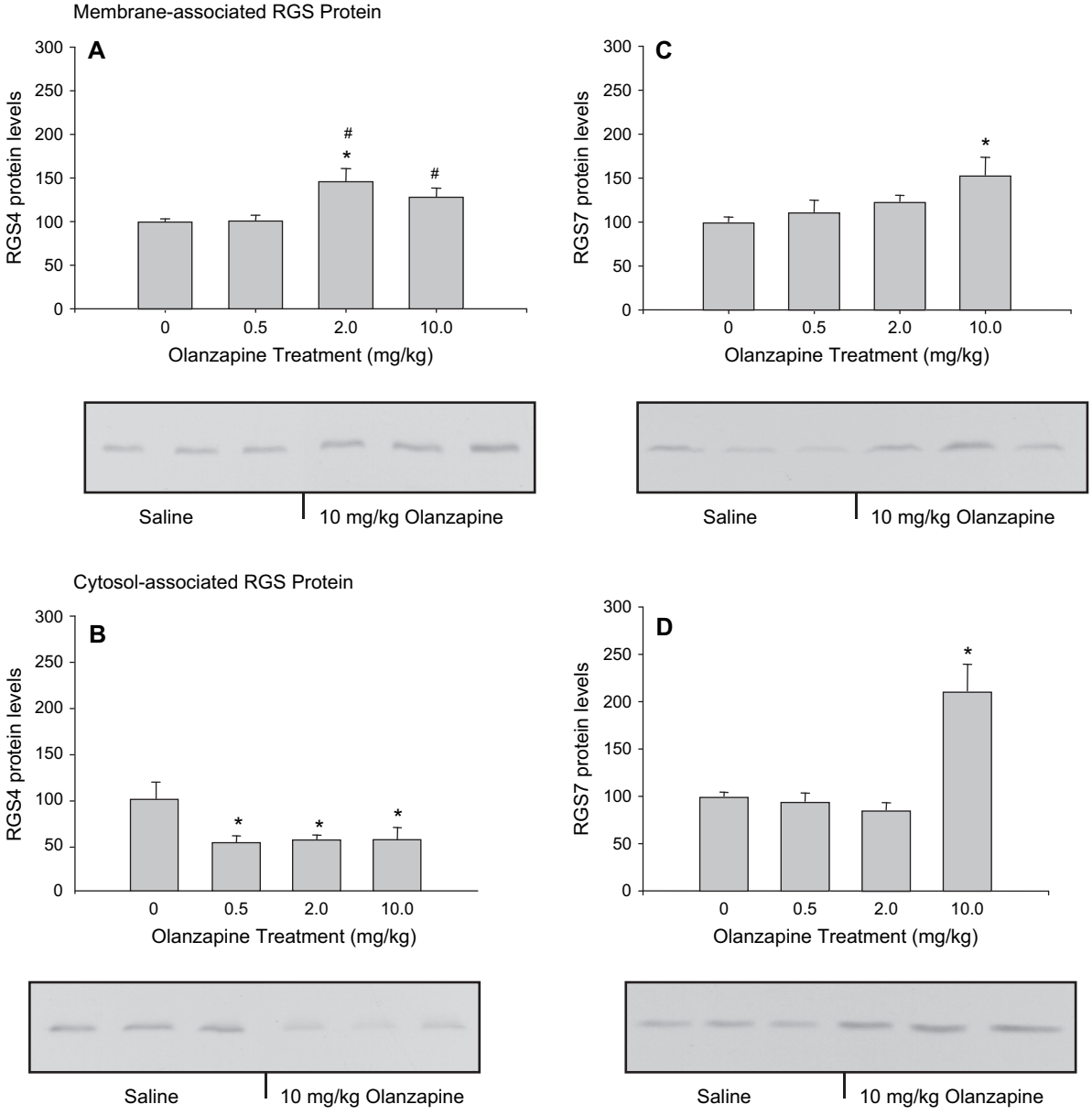

Fig. 6. Chronic olanzapine differentially altered RGS4 and RGS7 protein levels in the frontal cortex. RGS4 levels in the membrane fraction (A) were increased by daily treatment with 2.0 and 10.0 mg/kg olanzapine. The levels of RGS4 in the cytosol (B) were reduced by approximately half with all doses 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 $p < 0.05$.

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 [I^{125}] DOI labeled 5-HT_{2A} receptors in the frontal cortex of rats treated with 10 mg/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

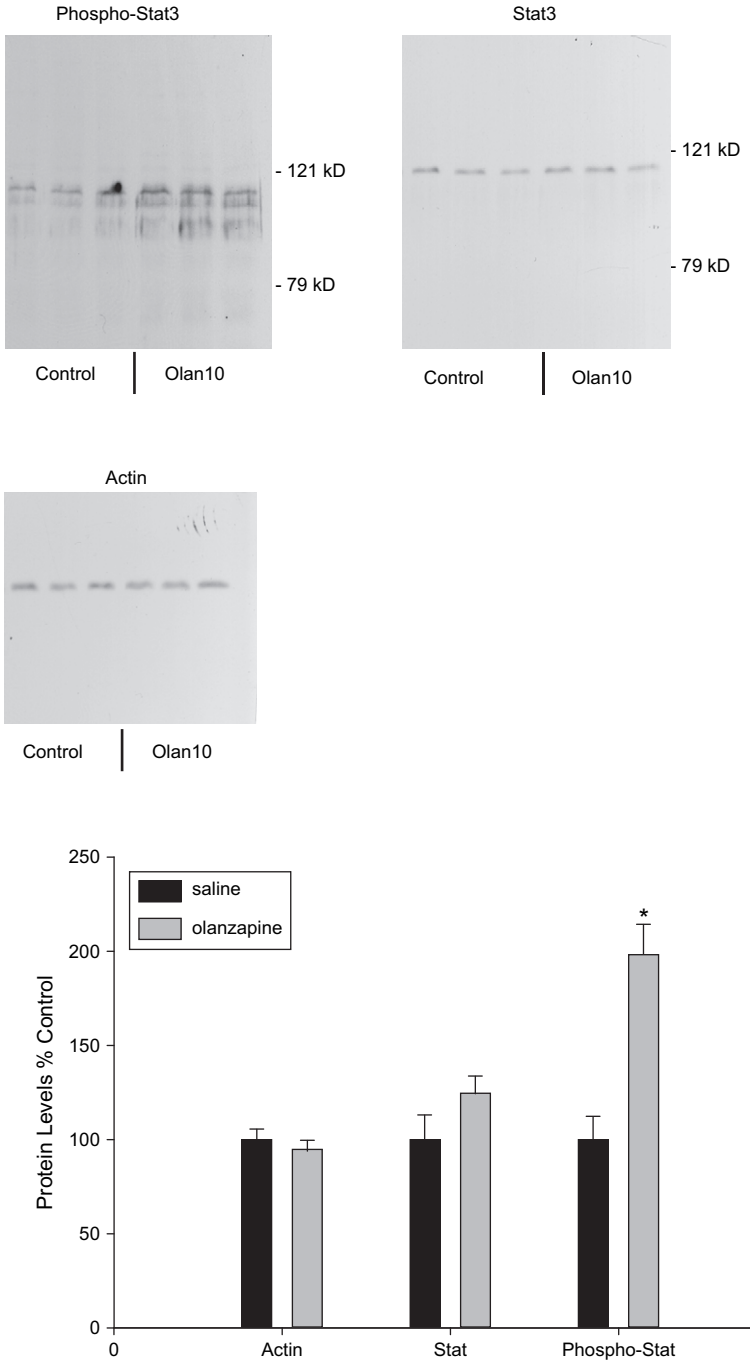

Fig. 7. Chronic olanzapine increased Stat3 activity in the frontal cortex. The levels of phosphorylated Stat 3 were significantly increased by daily treatments of 10 mg/kg olanzapine (Olan 10). Stat protein levels were not altered by olanzapine. Actin protein levels were used to verify equal loading of lanes on the SDS-PAGE gel. *Indicates significantly different from saline-treated control rats at $p < 0.05$.

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 $G_{\alpha 11}$ 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 GTPγS,

a non-hydrolysable GTP analog is used to activate G proteins as in our PLC assay, 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 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, $G_{\alpha i}$ protein but not $G_{\alpha q}$ protein expression increased association of RGS4 with the cell membrane. Increased $G_{\alpha o}$ but not $G_{\alpha q}$ protein expression increased palmitoylation and membrane association of RGS7 (Takida et al., 2005). $G_{\alpha 11}$ 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 $G_{\beta 5}$ 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 $G_{\beta 5}$ could increase the levels of RGS7 in the cytoplasm. RGS4 does not bind to 14-3-3 or $G_{\beta 5}$ 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 $G_{\alpha q}$ and $G_{\alpha 11}$ proteins increased with 10 mg/kg olanzapine treatments, the cytosolic levels of these proteins were unchanged. Our previous study in transgenic rats over-expressing $G_{\alpha q}$ protein showed a similar increase in only the membrane-associated fraction of $G_{\alpha q}$ 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 $G_{\alpha q/11}$ proteins. The constant levels of $G_{\alpha q/11}$ protein in the cytosol in these models suggest that the levels of $G_{\alpha q/11}$ in the cytosol are statically regulated while membrane-associated $G_{\alpha q/11}$ proteins are dynamic and follow expression levels of the proteins. An increase in $G_{\alpha q/11}$ 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 $G_{\alpha q/11}$ proteins could not contribute to the desensitization response caused by chronic olanzapine. Other studies in our laboratory suggest that phosphorylation of $G_{\alpha 11}$ 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 $G_{\alpha 11}$ protein occurs with chronic olanzapine treatment as well.

Not all of the G proteins examined after olanzapine treatment showed an increased expression. $G_{\beta 1-4}$ 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 $G_{\alpha q}$, $G_{\alpha 11}$, 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 [^{125}I]-(±)-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.

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