Activation of the JAK-STAT pathway by olanzapine is necessary for desensitization of serotonin2A receptor-stimulated phospholipase C signaling in rat frontal cortex but not serotonin2A receptor-stimulated hormone release

Rakesh K. Singh, **Cuihong Jia**, **Francesca Garcia**, **Gonzalo A. Carrasco**, **George Battaglia**, and **Nancy A. Muma**

Department of Pharmacology and Toxicology, School of Pharmacy, University of Kansas, 1251 Wescoe Hall Drive, 5064 Malott Hall, Lawrence Kansas 66045 (RKS, GAC, NAM) Loyola University Chicago, Stritch School of Medicine, 2160 South First Avenue, Maywood, Illinois 60153 (CJ, GB)

Abstract

Chronic treatment with olanzapine causes desensitization of serotonin2A receptor signaling. The purpose of the current study is to further understand the mechanisms underlying this desensitization response of serotonin2A receptor signaling in vivo. We now report that desensitization of serotonin2A receptor stimulated-phospholipase C activity in rat frontal cortex induced by olanzapine is dependent on activation of the JAK-STAT pathway. Olanzapine treatment for 7 days significantly increased the levels of the regulator of G protein signaling (RGS7) protein, RGS7 mRNA levels, and activation of JAK2 in rat frontal cortex. Pretreatment with a JAK2 inhibitor AG490, significantly attenuated the olanzapine-induced reductions in serotonin2A receptor-stimulated phospholipase C activity and prevented the olanzapine-induced increases in RGS7 mRNA and protein levels. In contrast, inhibition of the JAK-STAT pathway with AG490 did not reverse the olanzapine-induced desensitization of the serotonin2A receptor pathway in the hypothalamic paraventricular nucleus mediating increases in plasma hormone levels. AG490 dose-dependently inhibited serotonin2A receptor-stimulated oxytocin and corticosterone release. 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 serotonin2A receptor-stimulated phospholipase C activity in the frontal cortex but not serotonin2A receptor-stimulated hormone release.

Introduction

Serotonin 2A (5-HT$_{2A}$) receptors are involved 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 and in vivo (Zhang and Bymaster, 1999). Although atypical antipsychotics bind to diverse population of receptors (Roth, et al., 2004;Zhang and Bymaster, 1999), the 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). Further evidence for the involvement of 5-HT$_{2A}$ receptors in the mechanisms of action of atypical antipsychotics comes from reports of polymorphisms in the promoter and coding regions of the 5-HT$_{2A}$ receptor gene. Schizophrenics with this polymorphism are reported to respond poorly to clozapine treatment suggesting that genetic variation at 5-HT$_{2A}$ receptors

Correspondence to: Nancy A. Muma.

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). By determining the mechanisms by which atypical antipsychotics desensitize 5-HT$_{2A}$ receptor signaling, we may uncover new targets for drug manipulation.

Atypical antipsychotics reduce the levels of ACTH and cortisol in schizophrenic patients (Cohrs, et al., 2006;Hatzimanolis, et al., 1998;Scheepers, et al., 2001b), an effect that would be mediated by antagonism of 5-HT$_{2A}$ receptors in hypothalamic neuroendocrine cells. 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 (Hatzimanolis et al., 1998). In fact, we previously demonstrated that 5-HT$_{2A}$ receptors in the hypothalamic paraventricular nucleus (PVN) mediate the neuroendocrine responses to a peripheral injection of the 5-HT$_{2A/2C}$ receptor agonist (-)-1-(2,5-dimethoxy-4-iodophenyl)2-aminopropane (DOI); intra-PVN 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).

Several studies demonstrate that chronic administration of atypical antipsychotics causes the desensitization and down-regulation of central 5-HT$_{2A}$ receptor signaling (Blackshear and Sanders-Bush, 1982), however, the molecular mechanisms underlying these changes are not understood. 5-HT$_{2A}$ receptors are coupled through G$_{q/11}$ proteins to phospholipase C (PLC) (Roth, et al., 1998). Upon activation of PLC, hydrolysis of phosphatidylinositol 4,5-bisphosphate generates diacylglycerol and inositol 1,4,5-trisphosphate. 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) (Xu, et al., 1999).

Activation of 5-HT$_{2A}$ receptors also activates the JAK-STAT pathway. 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). We previously reported that daily treatment of rats with olanzapine for 7 days causes desensitization of 5-HT$_{2A}$ receptor signaling accompanied by activation of STAT3 and increases in RGS7 protein levels (Muma, et al., 2007). The JAK-STAT pathway regulates expression of a number of transcription factors including c-Fos, c-Jun and c-Myc (Burysek, et al., 2002;Cattaneo, et al., 1999) which can then stimulate expression of select genes. Consistent with these reports, olanzapine treatment in cell culture increases phospho-STAT binding to the putative promoter region of RGS7, increases RGS7 mRNA levels and membrane-associated RGS7 protein levels and causes JAK-STAT dependent desensitization (Singh et al., 2007; Singh, et al., in press). We hypothesize that the increased membrane-associated RGS7 protein can then increase hydrolysis of activated G$_{\alpha q/11}$ and contribute to the desensitization of 5-HT$_{2A}$ receptor signaling. However, whether olanzapine-induced activation of the JAK-STAT pathway has any direct impact on desensitization of 5-HT$_{2A}$ receptor signaling in vivo is currently unknown.

Here, we investigate 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 via measurements of PLC activity and in the PVN via hormone release. Furthermore, we also monitored the effect of AG490 on olanzapine-induced increases in RGS7 protein levels in PVN and the levels of RGS7 mRNA and protein in the frontal cortex.

Methods and Materials

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 were 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 Toronto 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 or 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 s.c. for the challenge injections.

Experimental procedures

Rats were randomly assigned to the 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 and handled for several days to minimize stress. Once a day for seven days, rats were first injected with vehicle (50% DMSO s.c.) 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, i.p.) or 10 mg/kg olanzapine (i.p.). Twenty-four hr after the last injection of vehicle 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.) and were sacrifice 30 min later. 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 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. The PLC activity assay was performed as described previously (Damjanoska, et al., 2004). 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 $13,600 \times g$. 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 bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

Radioimmunoassay of hormones

Plasma concentrations of oxytocin, ACTH, and corticosterone were determined by radioimmunoassay as described previously (Li, et al., 1993). We used this assay to measure the activity of 5-HT$_{2A}$ receptors in the PVN because we previously demonstrated that activation

of 5-HT$_{2A}$ receptors in this hypothalamic nucleus stimulates the release on oxytocin, ACTH and corticosterone (Zhang, et al., 2002).

Immunoblot analyses of RGS7, pJAK2, and JAK2 proteins

Tissue preparation—Frontal cortex and PVN from the treatment groups that received 1 mg/kg DOI injection were used for the measurement of pJAK2, JAK2 and RGS7 proteins. The PVN was dissected from a 700 µm coronal brain section obtained using a cryostat ($-10°C$) as previously described. 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 $20,000 \times g$ 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 blot analyses—Equal amounts of protein from vehicle-control and drug-treated samples were separated on 10% SDS polyacrylamide gels. Non-specific binding was blocked either in Tris-buffered saline (TBS) containing 5% (w/v) nonfat dry milk with 0.1% Tween 20 (TBST) or in phosphate buffered saline (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°C 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 groups were normalized to vehicle-treated groups 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 ratios at 260/280 nm were determined. Quality of RNA was further assessed on 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.

Real-Time PCR

The GAPDH (sense 5'-tggagtctactggcgtcttcac-3'; antisense 5'- ggcatggactgtggt catga-3') and RGS7 (sense 5'-gaagatgagttgcaccgacaga-3'; antisense 5'-ggtctttcagtgcct catccat-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 $2^{mean(ΔΔCT)}$, and the relative degree of response is calculated by $2^{-mean(ΔΔCT)}$. 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. RT-PCR, western blot data, and the PLC activity assays were 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. RGS7 and phospho-JAK2 levels in the PVN of rats treated with 10 mg/kg olanzapine were compared to vehicle-treated controls using a Student's *t*-test.

Results

Phosphorylation of JAK2 in frontal cortex was blocked by AG490 treatment

To verify that the JAK-STAT signaling pathway in frontal cortex is stimulated treatment with olanzapine as previously demonstrate (Muma et al., 2007), we first measured the phosphorylation of JAK2. 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 1. The total JAK2 protein level was not significantly altered with olanzapine treatment. Next, we determined whether the JAK2 inhibitor AG490 indeed inhibited JAK2 activation via phosphorylation. The olanzapine-induced increases in phosphorylation of JAK2 were significantly reduced to basal levels by pre-injections of AG490 at 10 mg/kg. As a control, we also measured the effects of treatment with AG490 alone and found that 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 ($F_{(1,15)} = 31.28$, $p = 0.001$).

Inhibition of the JAK-STAT pathway attenuated the olanzapine-induced inhibition of 5-HT$_{2A}$ receptor-mediated PLC activity in frontal cortex

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, in the current study, 10mg/kg olanzapine treatment for 7 days caused a 68% decrease (*p<0.01) in 5-HT-stimulated PLC activity in the frontal cortex (Figure 2A). As a control, PLC activity was examined in rats treated with AG490 alone; PLC activity was not affected by AG490 treatment alone. However, the decrease in 5-HT-stimulated PLC activity induced by olanzapine was significantly attenuated (*p<0.05) in rats injected with AG490 and olanzapine at 10 mg/kg compared to rats injected with vehicle and olanzapine, suggesting that the olanzapine-induced desensitization of PLC activity in rat frontal cortex is dependent on activation of the JAK2-STAT3 pathway. 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 had no effect (p> 0.05) on GTPγS-stimulated PLC activity (Figure 2B).

Olanzapine-induced increases in RGS7 mRNA and protein levels in frontal cortex were blocked by pre-treatment with AG490

Our previous studies demonstrated that olanzapine treatment increases RGS7 protein levels in rat frontal cortex (Muma et al., 2007) and that the olanzapine-induced increases RGS7 protein and mRNA levels in cells in culture are dependent on activation of the JAK2-STAT3 pathway (Singh et al., in press). We now wanted to determine if RGS7 mRNA levels are increased in rat frontal cortex and if the increases in RGS7 protein and mRNA are dependent on activation of the JAK2-STAT3 pathway. Olanzapine injections for 7 days significantly increased mRNA (*p<0.05) and protein (*p<0.05) levels of RGS7 as shown in Figure 3A and 3B. To control for the effects of AG490, we examined the effects of AG490 alone on RGS7 expression. Both mRNA and protein levels of RGS7 were not affected by AG490 treatment alone. However, 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). These results suggest that the increased expression of RGS7 induced by olanzapine is dependent on the JAK2-STAT3 pathway in rat frontal cortex as in cells in culture.

Olanzapine-induced desensitization of 5-HT$_{2A}$ receptor signaling in PVN was not reduced by AG490

Basal plasma ACTH, corticosterone, and oxytocin levels were not significantly altered after seven daily injections of AG490 and olanzapine. DOI-challenge produced a significant (*p<0.01) increase (1,257%) in plasma oxytocin levels in vehicle-pretreated rats, in 2 mg/kg AG490 pre-treated rats (853%, *p<0.01), and in 10 mg/kg AG490 pre-treated rats (449%, *p<0.01) compared to saline-challenged rats. However, AG490 -pretreatment produced a dose-dependent inhibition of oxytocin release in rats. Olanzapine treatment completely blocked the DOI-induced increases in oxytocin release as compared to vehicle-treated rats (Figure 4A). Although, a trend towards reversal of the olanzapine-inhibited oxytocin level was observed at the high dose of AG490 (10 mg/kg), it was not statistically significant. 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 and olanzapine ($F_{(1,81)}$= 28.12, P<0.0001), DOI and AG490 ($F_{(2,81)}$= 63.57, P<0.0001), and olanzapine and AG490 ($F_{(2,81)}$= 165.69, P<0.0001) were also observed. Finally, there was significant interaction among DOI, olanzapine and AG490 ($F_{(2,81)}$= 65.83, P<0.0001).

ACTH response to DOI—A DOI-challenge produced a significant (*p<0.01) increase in plasma ACTH levels in vehicle-pretreated rats (1279%) and in 2 mg/kg AG490-pretreated rats (1296%) (*p<0.01), and in 10 mg/kg AG490 injected rats (1032%) (*p<0.01) compared to saline-challenged rats (Fig.4B). Olanzapine treatment significantly (38%,*p<0.01) attenuated the DOI-stimulated increase in ACTH release as compared to vehicle treated rats (Figure 4B). However, AG490 treatment had no significant effect on ACTH levels in rats treated with olanzapine compared to the respective 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 and olanzapine $(F_{(1,66)} = 16.46, P<0.001)$, DOI and AG490 $(F_{(2,66)} = 99.68, P<0.0001)$, and olanzapine and AG490 $(F_{(2,66)} = 1897.87, P<0.0001)$ was also observed. There was also a significant interaction among DOI and olanzapine and 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 in vehicle-pretreated rats (685%) and in 2 mg/kg AG490-pretreated rats (524%) (*p<0.01), and in 10 mg/kg AG490 injected rats (406%) (*p<0.01) compared to saline-challenged rats (Fig.4C). Olanzapine treatment significantly (54%,*p<0.01) attenuated the DOI-stimulated increase in corticosterone release as compared to vehicle-treated rats (Figure 4C). 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 corticosterone $(F_{(1,82)} = 43.57, P<0.0001)$, a main 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 and olanzapine $(F_{(1,82)} = 29.63, P<0.001)$, DOI and AG490 $(F_{(2,82)} = 62.98, P<0.0001)$, and olanzapine and AG490 $(F_{(2,82)} = 164.50, P<0.0001)$ was also observed. Finally, there was a significant interaction between DOI, olanzapine and AG490 $(F_{(2,82)} = 68.01, P<0.0001)$.

Olanzapine increased JAK2 phosphorylation in the PVN

Olanzapine treatment (10 mg/kg, for 7 days) significantly (p<0.01) increased ($184 \pm 11.9\%$ of control levels) the membrane-associated levels of phosphorylated JAK2 in the PVN (Fig. 5). As shown in Fig 5, total JAK2 protein levels in the PVN were not altered by chronic olanzapine treatment.

Olanzapine increased RGS7 protein levels in the PVN

As shown in Fig 6, membrane-associated RGS7 protein levels in the PVN in olanzapine treated rats were significantly increased to over twice ($208 \pm 19.2\%$ of control levels) the levels in vehicle treated rats ($t = -7.9, p < 0.0001$). Actin was used as a loading control.

Discussion

Although, atypical antipsychotics bind to a number of different neurotransmitter receptors (Zhang and Bymaster, 1999;Roth et al., 2004), their therapeutic benefits are attributed, in part, to their ability to desensitize 5-HT$_{2A}$ receptor signaling (Goyer et al., 1996). Previous studies have shown that 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 of 5-HT$_{2A}$ receptors 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 cell culture, olanzapine causes desensitization of 5-HT$_{2A}$ receptor-stimulated PLC signaling, increases RGS7 protein expression and activates the JAK-STAT pathway (Muma, et al., 2007;Singh, et al., 2007). Furthermore, we found that in A1A1v cells JAK-STAT signaling is necessary for approximately half of the desensitization response (Singh et al., in press).

Consistent with our previous findings in cultured A1A1v cells, we now find that the olanzapine-induced desensitization of 5-HT$_{2A}$ receptor-mediated PLC activity in the rat frontal cortex is dependent on activation of the JAK-STAT signaling pathway. Indeed, AG490-pretreatment that inhibited the olanzapine-induced activation of JAK2 in frontal cortex, prevented the olanzapine-induced desensitization of 5-HT$_{2A}$ receptor signaling and attenuated the olanzapine-induced increases in RGS7 mRNA and protein levels in frontal cortex. We previously reported that increased RGS7 protein expression in response to olanzapine treatment

is dependent on JAK-STAT signaling in A1A1v cells (Singh et al., 2007). The current data suggest that activation of the JAK-STAT pathway is necessary for the complete olanzapine-induced desensitization of 5-HT$_{2A}$ receptor signaling in frontal cortex as well.

RGS proteins can reduce G-protein-mediated signaling by their ability to increase the intrinsic guanosine triphosphatase (GTPase) activity of heterotrimeric G proteins or by blocking the interaction of Gα proteins with effectors. The GTPase accelerating protein (GAP) activity enhances G protein deactivation and promotes desensitization (Dohlman and Thorner, 1997). 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.

Previous studies have reported down-regulation of RGS4 expression in the frontal cortex of schizophrenic patients (Mirnics et al., 2001; Gu et al., 2007). However, none of these studies have identified alterations in RGS7 expression. 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. Our real-time PCR data suggests that the increase in RGS7 levels by olanzapine is mediated by a direct increase in RGS7 mRNA via activation of JAK-STAT pathway. In our previous studies, we identified a STAT3 consensus binding element located 2.34kb upstream of transcription start site that binds strongly with STAT3 in response to olanzapine treatment (Singh et al., in press). 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.

Alternatively, interaction of RGS7 with G$_{\beta 5}$ (Zhang and Simonds, 2000) 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). 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, or increasing the stability of RGS7 protein and could mediate, at least in part, the olanzapine-induced regulation of RGS7 GAP activity.

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), and Akt/PKB and P38 pathways in PC12 cells (Lu, et al., 2004). 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% attenuation of the olanzapine-induced reductions in PLC activity in the frontal cortex 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 the negative symptomatology in schizophrenia (Shirayama, et al., 2002;Walker, et al., 2002). Studies have also demonstrated that the atypical antipsychotic-induced decrease in cortisol levels is associated with an improvement in psychopathology (Hatzimanolis, et al., 1998)(Markianos, et al., 2001). The olanzapine-induced decrease in plasma levels of oxytocin, ACTH, and corticosterone in this study are consistent with previous reports (Scheepers, et al., 2001a;Tepavcevic, et al., 1994) and suggest that chronic olanzapine-treatment produced a functional desensitization of 5-HT$_{2A}$ receptors in PVN as well. 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 signaling pathway. Unfortunately, we are not able to measure 5-HT$_{2A}$ receptor-stimulated PLC activity in the PVN to directly compare to the frontal cortex; due to the small size of the PVN. We have previously reported that the neuroendocrine effects of DOI are mediated exclusively by activation of 5-HT$_{2A}$ receptors (but not 5-HT$_{2C}$ receptors) in PVN (Zhang et al., 2002). Here, we found an apparent dose-dependent inhibitory effect of AG490-pretreatment on 5-HT$_{2A}$ receptor stimulated oxytocin and corticosterone plasma levels whereas ACTH levels were not affected. There are other examples of dissociation of the regulation of hormone responses to 5-HT receptor stimulation such as in oxytocin and ACTH responses to fluoxetine or estradiol (D'Souza, et al., 2004;Damjanoska, et al., 2003). These results suggest that the JAK-STAT signaling pathway could be involved in either the synthesis or release of these hormones. Further experiments, studying the expression of JAK-STAT proteins in either oxytocin and/or CRF containing cells of the PVN could shed light in the mechanisms by which JAK-STAT dose-dependently regulate the 5-HT$_{2A}$-mediated release of oxytocin and corticosterone. Furthermore, differences in the relative levels of RGS7 and RGS4 in frontal cortex compared to the PVN could also contribute to difference in the effects of the JAK-STAT inhibitor AG490 on the 5-HT$_{2A}$ receptor stimulated responses in these brain regions. Another possible difference between the desensitization responses could be different PLC isoforms mediating the signaling pathways.

In summary, this study demonstrates that olanzapine-induced desensitization of 5-HT$_{2A}$ receptor-stimulated PLC signaling is dependent on increased expression of RGS7 protein via activation of the JAK-STAT pathway in rat frontal cortex as was previously demonstrated in a cortical cell line. However, olanzapine-induced desensitization of 5-HT$_{2A}$ receptors in neuroendocrine cells appears to be independent of JAK-STAT, suggesting either regional differences or differences in the regulation of pathways down-stream from 5-HT$_{2A}$ receptors by atypical antipsychotics. Further studies are needed to determine the role of JAK-STAT signaling pathway in the regulation of plasma hormone levels.

Acknowledgments

Reference List

Arranz MJ, Collier DA, Munro J, Sham P, Kirov G, Sodhi M, Roberts G, Price J, Kerwin RW. Analysis of a structural polymorphism in the 5-HT2A receptor and clinical response to clozapine. Neurosci Lett 1996;217:177–178. [PubMed: 8916101]

Banes AK, Shaw SM, Tawfik A, Patel BP, Ogbi S, Fulton D, Marrero MB. Activation of the JAK/STAT pathway in vascular smooth muscle by serotonin. Am J Physiol Cell Physiol 2005;288:C805–C812. [PubMed: 15601754]

Benzing T, Kottgen M, Johnson M, Schermer B, Zentgraf H, Walz G, Kim E. Interaction of 14–3–3 protein with regulator of G protein signaling 7 is dynamically regulated by tumor necrosis factor-alpha. J Biol Chem 2002;277:32954–32962. [PubMed: 12077120]

Benzing T, Yaffe MB, Arnould T, Sellin L, Schermer B, Schilling B, Schreiber R, Kunzelmann K, Leparc GG, Kim E, Walz G. 14–3–3 interacts with regulator of G protein signaling proteins and modulates their activity. J Biol Chem 2000;275:28167–28172. [PubMed: 10862767]

Blackshear MA, Sanders-Bush E. Serotonin receptor sensitivity after acute and chronic treatment with mianserin. J Pharmacol Exp Ther 1982;221:303–308. [PubMed: 7077527]

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]

Cohrs S, Roher C, Jordan W, Meier A, Huether G, Wuttke W, Ruther E, Rodenbeck A. The atypical antipsychotics olanzapine and quetiapine, but not haloperidol, reduce ACTH and cortisol secretion in healthy subjects. Psychopharmacology (Berl) 2006;185:11–18. [PubMed: 16432682]

D'Souza DN, Zhang Y, Damjanoska KJ, Carrasco GA, Sullivan NR, Garcia F, Battaglia G, Doncarlos LL, Muma NA, Van de Kar LD. Estrogen reduces serotonin-1A receptor-mediated oxytocin release and Galpha(i/o/z) proteins in the hypothalamus of ovariectomized rats. Neuroendocrinology 2004;80:31–41. [PubMed: 15385710]

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-hydroxytryptamine (2A) receptor signaling in frontal cortex, oxytocin- and corticotropin-releasing factor-containing neurons in rat paraventricular nucleus. J Pharmacol Exp Ther 2003;306:563–571. [PubMed: 12721328]

Dohlman HG, Thorner J. RGS proteins and signaling by heterotrimeric G proteins. J Biol Chem 1997;272:3871–3874. [PubMed: 9064301]

Fuller RW, Snoddy HD. Central dopamine receptors mediating pergolide-induced elevation of serum corticosterone in rats. Characterization by the use of antagonists. Neuropharmacology 1984;23:1389–1394. [PubMed: 6527743]

Fumagalli F, Frasca A, Sparta M, Drago F, Racagni G, Riva MA. Long-term exposure to the atypical antipsychotic olanzapine differently up-regulates extracellular signal-regulated kinases 1 and 2 phosphorylation in subcellular compartments of rat prefrontal cortex. Mol Pharmacol 2006;69:1366–1372. [PubMed: 16391238]

Goyer PF, Berridge MS, Morris ED, Semple WE, Compton-Toth BA, Schulz SC, Wong DF, Miraldi F, Meltzer HY. PET measurement of neuroreceptor occupancy by typical and atypical neuroleptics. J Nucl Med 1996;37:1122–1127. [PubMed: 8965181]

Gray JA, Roth BL. Paradoxical trafficking and regulation of 5-HT(2A) receptors by agonists and antagonists. Brain Res Bull 2001;56:441–451. [PubMed: 11750789]

Guillet-Deniau I, Burnol AF, Girard J. Identification and localization of a skeletal muscle secrotonin 5-HT2A receptor coupled to the Jak/STAT pathway. J Biol Chem 1997;272:14825–14829. [PubMed: 9169451]

Guo D, Dunbar JD, Yang CH, Pfeffer LM, Donner DB. Induction of Jak/STAT signaling by activation of the type 1 TNF receptor. J Immunol 1998;160:2742–2750. [PubMed: 9510175]

Hanley NR, Hensler JG. Mechanisms of ligand-induced desensitization of the 5-hydroxytryptamine(2A) receptor. J Pharmacol Exp Ther 2002;300:468–477. [PubMed: 11805206]

Hatzimanolis J, Lykouras L, Markianos M, Oulis P. Neurochemical variables in schizophrenic patients during switching from neuroleptics to clozapine. Prog Neuropsychopharmacol Biol Psychiatry 1998;22:1077–1085. [PubMed: 9829289]

Lefebvre H, Contesse V, Delarue C, Vaudry H, Kuhn JM. Serotonergic regulation of adrenocortical function. Horm Metab Res 1998;30:398–403. [PubMed: 9694569]

Li Q, Levy AD, Cabrera TM, Brownfield MS, Battaglia G, Van de Kar LD. Long-term fluoxetine, but not desipramine, inhibits the ACTH and oxytocin responses to the 5-HT1A agonist, 8-OH-DPAT, in male rats. Brain Res 1993;630:148–156. [PubMed: 8118681]

Lu XH, Bradley RJ, Dwyer DS. Olanzapine produces trophic effects in vitro and stimulates phosphorylation of Akt/PKB, ERK1/2, and the mitogen-activated protein kinase p38. Brain Res 2004;1011:58–68. [PubMed: 15140644]

Markianos M, Hatzimanolis J, Lykouras L. Neuroendocrine serotonergic and dopaminergic responsivity in male schizophrenic patients during treatment with neuroleptics and after switch to risperidone. Psychopharmacology (Berl) 2001;157:55–59. [PubMed: 11512043]

Masellis M, Basile V, Meltzer HY, Lieberman JA, Sevy S, Macciardi FM, Cola P, Howard A, Badri F, Nothen MM, Kalow W, Kennedy JL. Serotonin subtype 2 receptor genes and clinical response to clozapine in schizophrenia patients. Neuropsychopharmacology 1998;19:123–132. [PubMed: 9629566]

Muma NA, Singh RK, Vercillo MS, D'Souza DN, Zemaitaitis B, Garcia F, Damjanoska KJ, Zhang Y, Battaglia G, Van de Kar LD. Chronic olanzapine activates the Stat3 signal transduction pathway and alters expression of components of the 5-HT2A receptor signaling system in rat frontal cortex. Neuropharmacology 2007;53:552–562. [PubMed: 17675105]

Naughton M, Mulrooney JB, Leonard BE. A review of the role of serotonin receptors in psychiatric disorders. Hum Psychopharmacol 2000;15:397–415. [PubMed: 12404302]

Nichols DE, Frescas S, Marona-Lewicka D, Huang X, Roth BL, Gudelsky GA, Nash JF. 1-(2,5-Dimethoxy-4-(trifluoromethyl)phenyl)-2-aminopropane: a potent serotonin 5-HT2A/2C agonist. J Med Chem 1994;37:4346–4351. [PubMed: 7996545]

Roth BL, Berry SA, Kroeze WK, Willins DL, Kristiansen K. Serotonin 5-HT2A receptors: molecular biology and mechanisms of regulation. Crit Rev Neurobiol 1998;12:319–338. [PubMed: 10348614]

Roth BL, Sheffler DJ, Kroeze WK. Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia. Nat Rev Drug Discov 2004;3:353–359. [PubMed: 15060530]

Scheepers FE, Gespen de Wied CC, Kahn RS. The effect of olanzapine treatment on m-chlorophenylpiperazine-induced hormone release in schizophrenia. J Clin Psychopharmacol 2001b; 21:575–582. [PubMed: 11763004]

Scheepers FE, Gespen de Wied CC, Kahn RS. The effect of olanzapine treatment on m-chlorophenylpiperazine-induced hormone release in schizophrenia. J Clin Psychopharmacol 2001a; 21:575–582. [PubMed: 11763004]

Shirayama Y, Hashimoto K, Suzuki Y, Higuchi T. Correlation of plasma neurosteroid levels to the severity of negative symptoms in male patients with schizophrenia. Schizophr Res 2002;58:69–74. [PubMed: 12363392]

Singh RK, Shi J, Zemaitaitis BW, Muma NA. Olanzapine increases RGS7 protein expression via stimulation of the Janus tyrosine kinase-signal transducer and activator of transcription signaling cascade. J Pharmacol Exp Ther 2007;322:133–140. [PubMed: 17392403]

Tepavcevic D, Giljevic Z, Korsic M, Halimi S, Suchanek E, Jelic T, Aganovic I, Kozic B, Plavsic V. Effects of ritanserin, a novel serotonin-S2 receptor antagonist, on the secretion of pituitary hormones in normal humans 2. J Endocrinol Invest 1994;17:1–5. [PubMed: 8006323]

Tuomisto J, Mannisto P. Neurotransmitter regulation of anterior pituitary hormones. Pharmacol Rev 1985;37:249–332. [PubMed: 2869509]

Walker EF, Bonsall R, Walder DJ. Plasma hormones and catecholamine metabolites in monozygotic twins discordant for psychosis 1. Neuropsychiatry Neuropsychol Behav Neurol 2002;15:10–17. [PubMed: 11877547]

Wilcox CS, Aminoff MJ, Millar JG, Keenan J, Kremer M. Circulating levels of corticotrophin and cortisol after infusions of L-DOPA, dopamine and noradrenaline, in man. Clin Endocrinol (Oxf) 1975;4:191–198. [PubMed: 165915]

Xu X, Zeng W, Popov S, Berman DM, Davignon I, Yu K, Yowe D, Offermanns S, Muallem S, Wilkie TM. RGS proteins determine signaling specificity of Gq-coupled receptors. J Biol Chem 1999;274:3549–3556. [PubMed: 9920901]

Yu YW, Tsai SJ, Yang KH, Lin CH, Chen MC, Hong CJ. Evidence for an association between polymorphism in the serotonin-2A receptor variant (102T/C) and increment of N100 amplitude in schizophrenics treated with clozapine. Neuropsychobiology 2001;43:79–82. [PubMed: 11174050]

Zhang JH, Simonds WF. Copurification of brain G-protein beta5 with RGS6 and RGS7. J Neurosci 2000;20:RC59. [PubMed: 10648734]

Zhang W, Bymaster FP. The in vivo effects of olanzapine and other antipsychotic agents on receptor occupancy and antagonism of dopamine D1, D2, D3, 5HT2A and muscarinic receptors. Psychopharmacology (Berl) 1999;141:267–278. [PubMed: 10027508]

Zhang Y, Damjanoska KJ, Carrasco GA, Dudas B, D'Souza DN, Tetzlaff J, Garcia F, Hanley NR, Scripathirathan K, Petersen BR, Gray TS, Battaglia G, Muma NA, Van de Kar LD. Evidence that 5-HT2A receptors in the hypothalamic paraventricular nucleus mediate neuroendocrine responses to (-)DOI. J Neurosci 2002;22:9635–9642. [PubMed: 12417689]
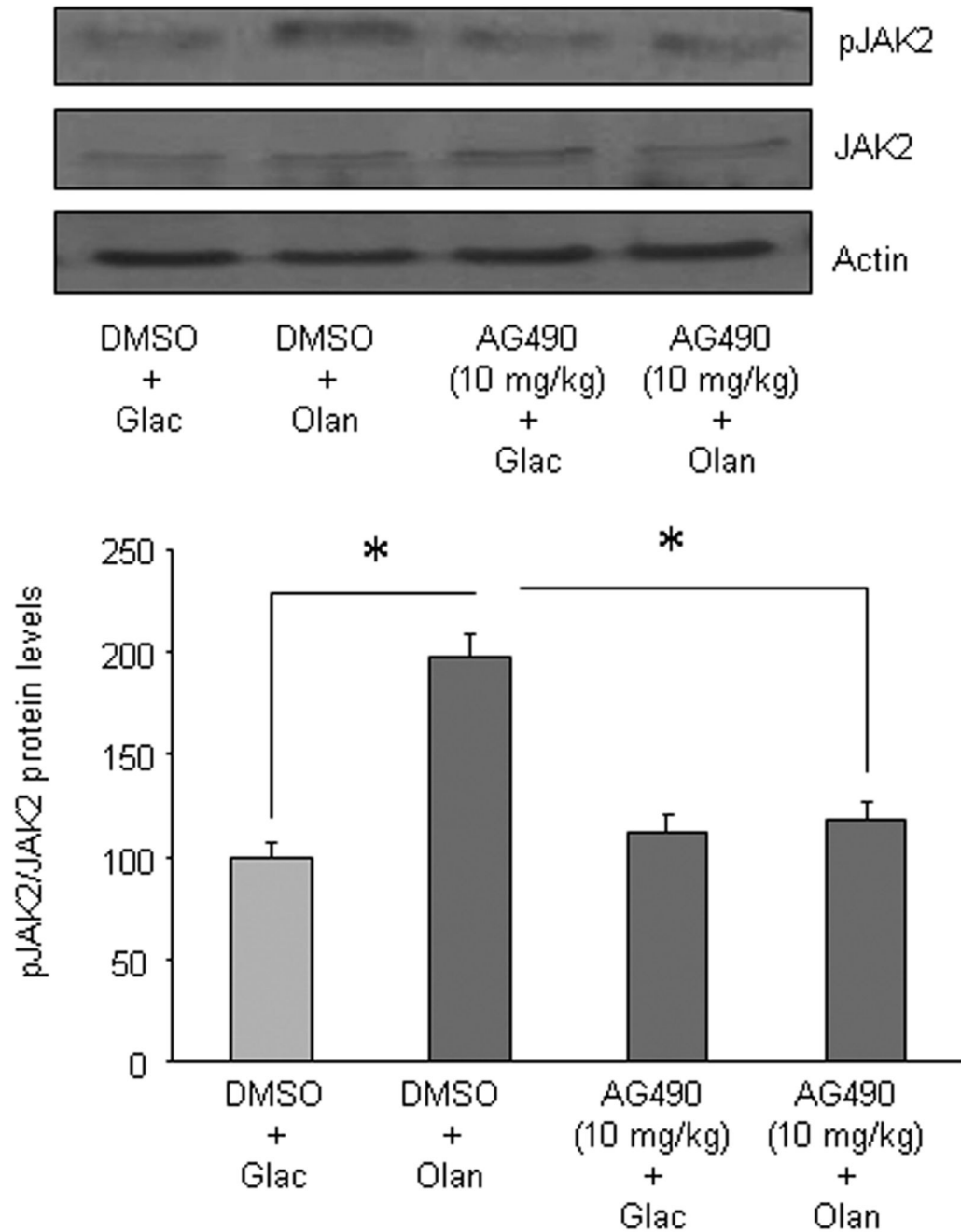
Figure 1.
Olanzapine treatment significantly increased (* p<0.05) phosphorylation of JAK2 in the frontal cortex. AG490 treatment alone had no effect on phosphorylation of JAK2, however, inhibition of the JAK-STAT pathway with AG490 pre-treatment significantly attenuated phosphorylation of JAK2. Total 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. There were four animals in each treatment group and the western blots were repeated three times.

Figure 2.
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. There were five animals used in each treatment group and the assay was performed three times with two replicates.
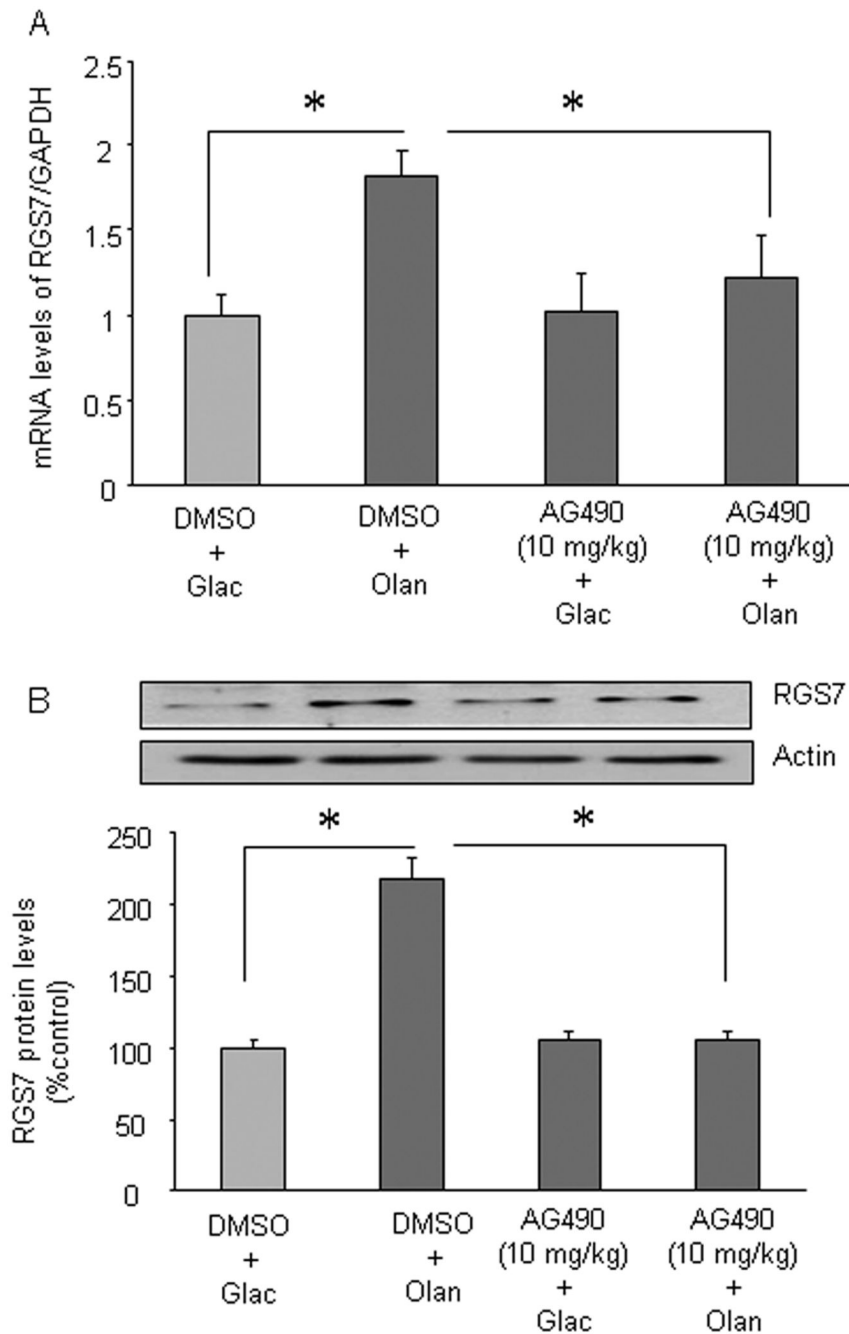
Figure 3.
Olanzapine treatment significantly (* p<0.05) increased RGS7 mRNA in the frontal cortex (A) and membrane-bound RGS7 protein levels (B) over vehicle-treated control rats. AG490 pretreatment completely blocked the olanzapine-induced increase in mRNA levels of RGS7. Olanzapine-induced increases 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. This assay was performed with four animals in each treatment group, three separate times and each time in triplicate.
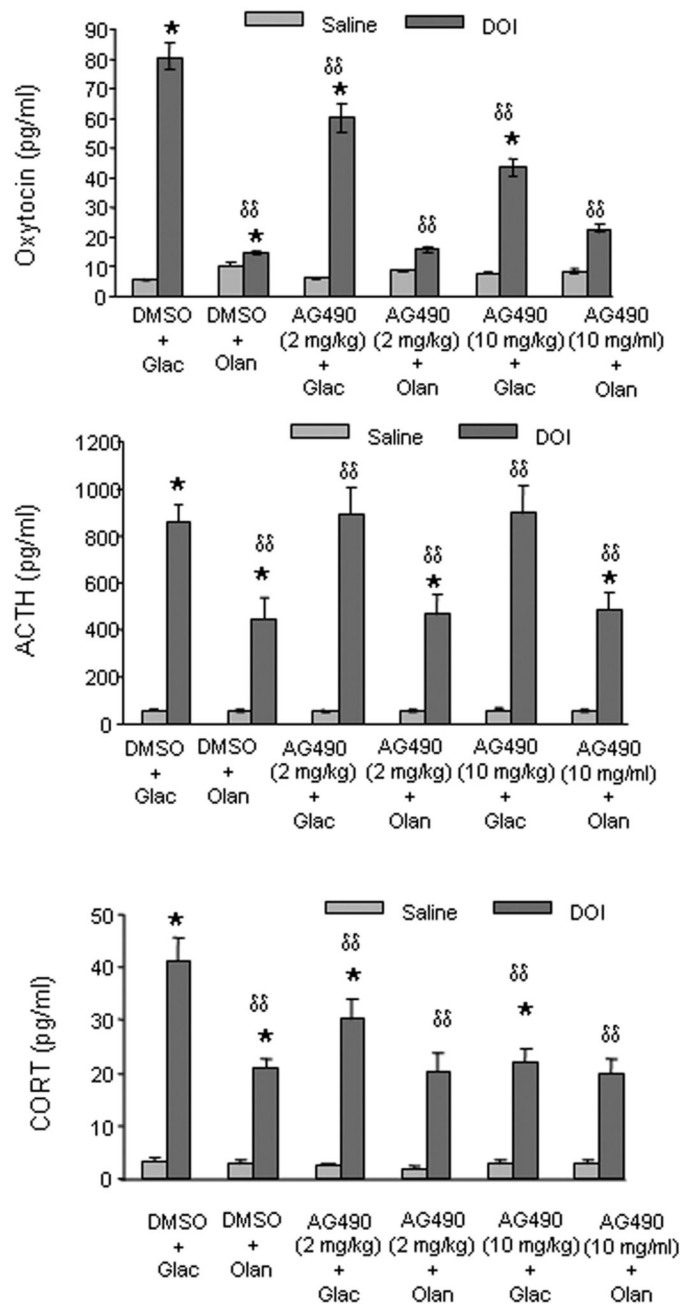
Figure 4.
Chronic treatment with olanzapine significantly attenuated DOI-stimulated hormone responses. Oxytocin (A), ACTH (B) and corticosterone (C) responses to a challenge with DOI 1 mg/ml at 30 min post-injection. The data represent the mean ± S.E.M. of eight rats per group. Basal plasma oxytocin, 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 δ δ, $p < 0.01$. There were eight animals in each treatment group and each sample was examined in quadruplicate.
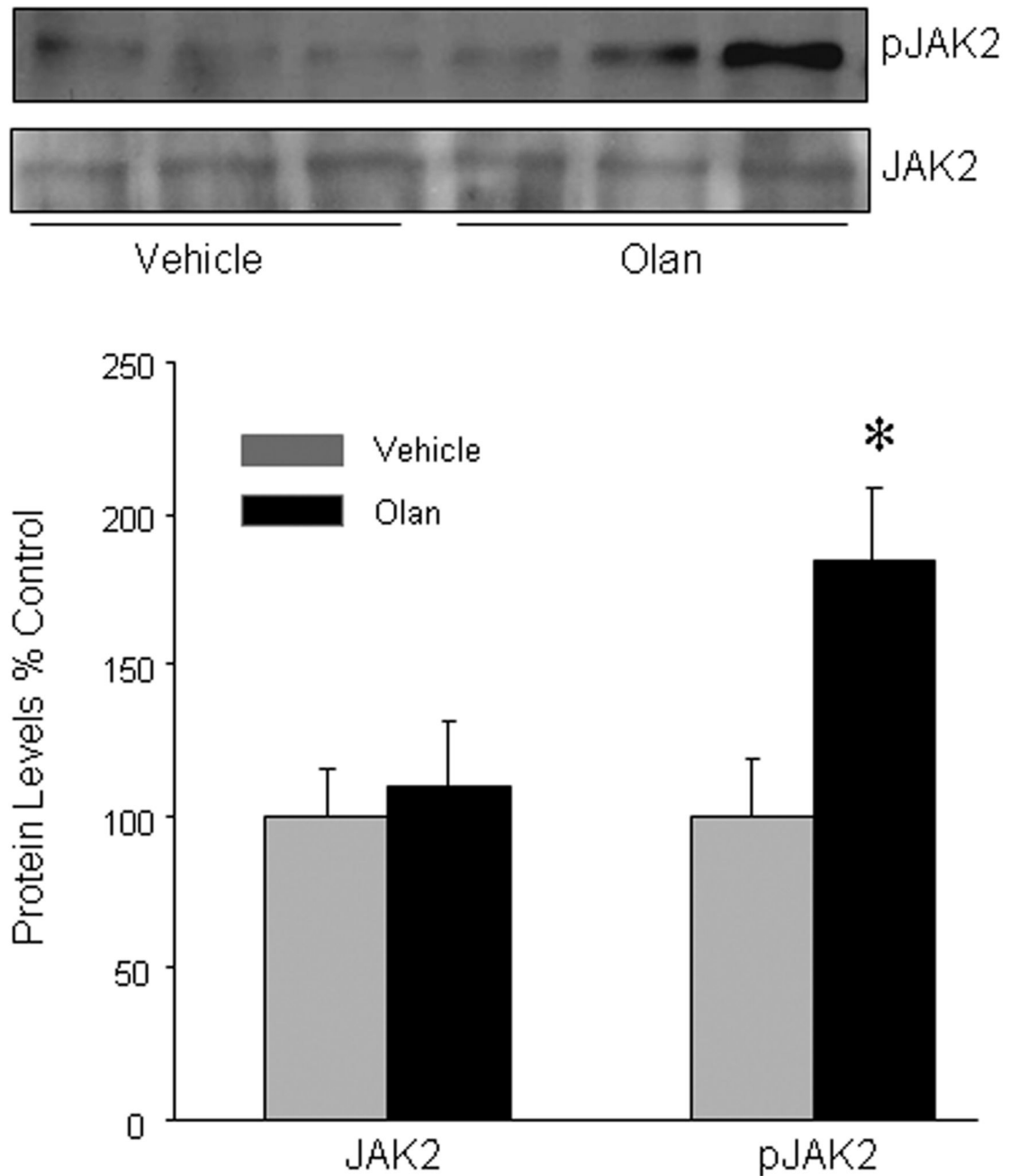
Figure 5.
Chronic olanzapine increased JAK2 phosphorylation in the PVN. The levels of phosphorylated JAK2 were significantly increased by daily treatments of 10 mg/kg olanzapine (Olan). Total JAK2 protein levels were not altered by olanzapine. *Indicates significantly different from vehicle-treated control rats at p<0.001. There were four animals in each treatment group and each western blot assay was performed three times.
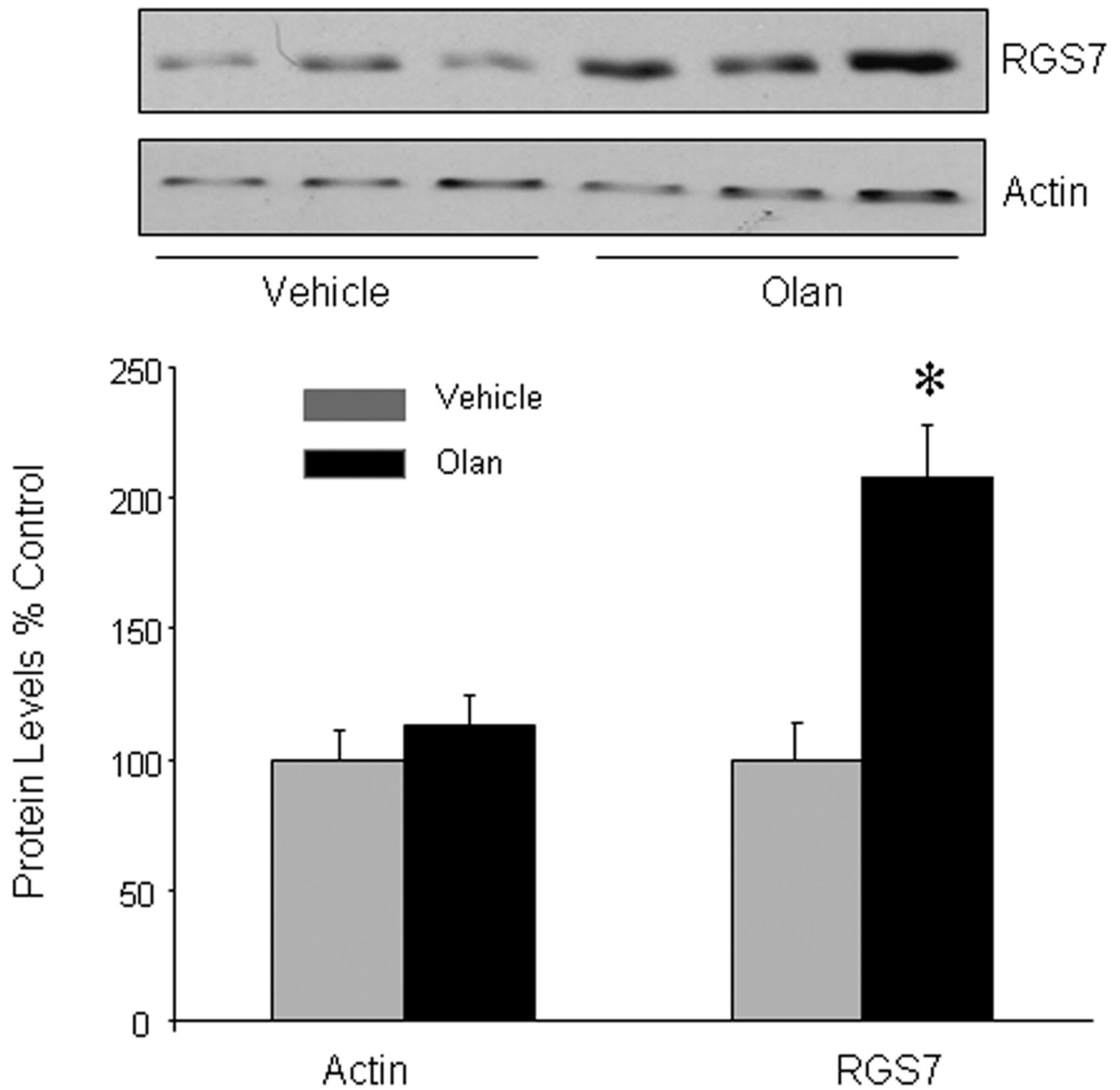
Figure 6.
Chronic olanzapine treatment increased RGS7 protein levels in the PVN. RGS7 levels in the membrane fraction of the PVN were increased by daily treatment with 10.0 mg/kg olanzapine. Actin was used to verify equal loading of lanes in the SDS PAGE gel. *Indicates significantly different from vehicle-treated control rats compared to 10 mg/kg olanzapine at $p < 0.001$. There were four animals in each treatment group and each western blot assay was performed three times.