Activation of the JAK-STAT pathway is necessary for desensitization of 5-HT$_{2A}$ receptor-stimulated phospholipase C signalling by olanzapine, clozapine and MDL 100907

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Abstract

We have previously demonstrated that olanzapine-induced desensitization of 5-HT$_{2A}$ receptor-stimulated phospholipase C (PLC) activity is associated with increases in RGS7 protein levels both in vivo and in cells in culture, and the increase in RGS7 is dependent on activation of the JAK-STAT pathway in cells in culture. In the present study, we found that desensitization of 5-HT$_{2A}$ receptor-stimulated PLC activity induced by olanzapine is dependent on activation of the JAK-STAT pathway. Similar to olanzapine, clozapine-induced desensitization of 5-HT$_{2A}$ receptor signalling is accompanied by increases in RGS7 and activation of JAK2. Treatment with the selective 5-HT$_{2A}$ receptor antagonist MDL 100907 also increased RGS7 protein levels and JAK2 activation. Using a JAK2 inhibitor AG490, we found that clozapine and MDL 100907-induced increases in RGS7 are dependent on activation of the JAK-STAT pathway. Olanzapine, clozapine, and MDL 100907 treatment increased mRNA levels of RGS7. Using a chromatin immunoprecipitation assay we found STAT3 binding to the putative RGS7 promoter region. Taken together, olanzapine-induced activation of the JAK-STAT pathway, and STAT3 binding to the RGS7 gene could underlie the increase in RGS7 mRNA which could subsequently increase protein expression. Furthermore, the increase in RGS7 protein could play a role in the desensitization of 5-HT$_{2A}$ receptor signalling by terminating the activated $G_{q/11}$ proteins more rapidly. Overall, our data suggest that the complete desensitization of 5-HT$_{2A}$ receptor-stimulated PLC activity by olanzapine, clozapine and MDL 100907 requires activation of the JAK-STAT pathway, which in turn increases RGS7 expression probably by direct transcriptional activity of STAT3.

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Introduction

Atypical antipsychotics like clozapine and olanzapine (dibenzopyridine derivatives) represent a relatively new generation of antipsychotics with fewer incidences of negative side-effects such as extra-pyramidal side-effects (EPS) (Meltzer, 1995). Although, atypical antipsychotics have a diverse receptor binding profile, 5-HT$_2$-receptor-based mechanisms have been postulated to play a critical role in the action of the atypical antipsychotic drugs (Willins et al., 1999). However, the process by which these drug–receptor interactions translate into long-term cellular adaptive changes resulting in antipsychotic efficacy is unknown.

Atypical antipsychotic drugs bind with high affinity to 5-HT$_{2A}$ receptors and desensitize 5-HT$_{2A}$ receptor signalling (Deutch et al., 1991; Meltzer, 1995; Seeger et al., 1995). Although, desensitization of 5-HT$_{2A}$ receptor signalling by atypical antipsychotics is reported to be associated with down-regulation and internalization (Willins et al., 1999); the molecular mechanisms that underlie these changes are not well understood. Activation of 5-HT$_{2A}$ receptors stimulates activation of $G_{q/11}$, which in turn activates effector
enzymes including phospholipase C (PLC). PLC catalyses release of diacylglycerol (DAG) and inositol triphosphate from phosphatidylinositol bisphosphate (PIP2). The released inositol phosphate (IP) can be measured as an index of 5-HT2A receptor signalling activity. In addition to these integral components of the receptor signalling system, regulators of G protein signalling (RGS) proteins modulate signalling of several G protein-coupled receptors (GPCR) (Koelle and Horvitz, 1996). RGS proteins can regulate G-protein signalling by functioning as GTPase-activating proteins (GAPs). GAP activity can hasten the termination of a signal upon removal of a stimulus, attenuate a signal either as a feedback inhibitor or in response to a second input, promote regulatory association of other proteins, or redirect signalling within a G-protein signalling network (Ross and Wilkie, 2000). RGS4 and RGS7 are highly enriched in various brain regions including frontal cortex and are reported to be GAPs for Gαq/11-associate 5-HT2A receptor signalling (Larminie et al., 2004). Khawaja et al. (1999) have extensively characterized cellular co-localization of RGS7 with Gαq/11 immunohistochemically throughout the adult rat brain and reported a heterogeneous and overlapping regional distribution (Khawaja et al., 1999).

We have previously reported that desensitization of 5-HT2A receptor signalling with chronic treatment of olanzapine is accompanied by activation of STAT3 and an increase in RGS7 protein levels in rat frontal cortex (Muma et al., 2007). In addition, we found that 24-h treatment with olanzapine causes desensitization of 5-HT2A receptor signalling and an increase in membrane-associated RGS7 protein that is dependent on activation of the JAK2-STAT3 pathway in A1A1v cells, a cell line endogenously expressing the 5-HT2A receptor signalling components (Singh et al., 2007). However, whether activation of the JAK-STAT pathway is necessary for olanzapine-induced desensitization and the mechanisms by which activation of the JAK-STAT pathway increase RGS7 protein are not currently known. Therefore, it is important to determine not only the role of the JAK-STAT pathway but also the mechanisms underlying up-regulation of RGS7 protein in response to antipsychotic treatment to help identify new targets for therapeutic intervention.

Increases in RGS7 protein levels could be mediated by several mechanisms, for example RGS7 binding to Gβ5 is reported to increase stability of each protein (Chen et al., 2003; Krumins et al., 2004) such that an increase in Gβ5 could increase RGS7 protein levels. Another possible mechanism is a direct increase in transcription of RGS7 thereby increasing RGS7 mRNA levels. We previously reported that inhibition of the JAK-STAT pathway completely blocked the increase in RGS7 protein levels by olanzapine (Singh et al., 2007). Although, transcriptional activity of STAT3 has been extensively reported for various genes (Aaronson and Horvath, 2002; Kisseleva et al., 2002; Schindler, 2002). STAT3 has not been identified as a transcription factor for RGS7. STAT3-mediated regulation of gene expression is associated with the presence of the consensus element TTCTN4GAA upstream of the transcription start site (Ehret et al., 2001; Wrighting and Andrews, 2006). Genomic sequence analysis of rat RGS7 revealed that there are multiple sets of TTCTN4GAA sequences. Thus, it is possible that STAT3 is a transcription factor for the RGS7 promoter.

Based on our previous reports that the olanzapine-induced increases in RGS7 protein levels are dependent on activation of the JAK-STAT pathway, we hypothesize that STAT3 is a transcription factor for RGS7 and is directly responsible for the increase in RGS7 protein levels by olanzapine treatment. In this study, we also examined whether another atypical antipsychotic, clozapine and a selective 5-HT2A receptor antagonist, MDL 100907 also activate the JAK-STAT pathway and increase RGS7 expression. Last, we determined whether activation of the JAK-STAT pathway is necessary for desensitization of 5-HT2A receptor signalling by these atypical antipsychotics.

**Method**

**Drugs**

Olanzapine and AG490 were purchased from Torrent Research Chemicals Inc. (ON, Canada). MDL 100907 was kindly provided by Sanofi Aventis (Bridgewater, NJ, USA). Clozapine was purchased from Tocris (Ellisville, MO, USA). Olanzapine was dissolved in 20% glacial acetic acid and the pH was adjusted to 6.5 with 10 M NaOH as described previously (Singh et al., 2007). AG490, MDL 100907 and clozapine were dissolved in 100% DMSO to obtain the desired concentration for individual treatments with each drug. (–)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) was purchased from Sigma-Aldrich (St Louis, MO, USA). HBSS mix (1× HBSS, 20 mM LiCl, 20 mM Hepes) was used to dissolve DOI. A stock solution of 100 μM clozapine was prepared in DMSO. For each treatment group an equal volume of drug or vehicle (10 μl), was added to 10 ml cell culture media. Furthermore, an equal volume of DMSO was added for each treatment group.
**Cell culture**

A1A1v cells, a cortical cell line, that endogenously expresses 5-HT$_{2A}$ receptors and its downstream components, were used for all experiments. Cells were grown in the charcoal-treated serum to diminish serotonin in the media 24 h before treatment with olanzapine, clozapine, or MDL 100907. Cells were treated with either vehicle or drugs for 24 h.

**Cell fractionation**

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

**Western analyses**

Equal amounts of protein from vehicle-control and drug-treated samples were separated on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane for 2 h at 100 V. Non-specific binding to the membranes was blocked either with TBS containing 5% (w/v) non-fat dry milk with 0.1% Tween-20 (TBST) or in PBS containing 5% (w/v) non-fat dry milk. The following primary antibodies were used: anti-RGS7 (1:2000; polyclonal antibody, Upstate Biotechnology Inc., Lake Placid, NY, USA), anti-phospho-JAK2 (1:1000; polyclonal antibody; Affinity Bioreagent, Golden, CO, USA), anti-JAK2 (1:5000; polyclonal antibody, Upstate Biotechnology Inc.), and anti-actin (1:10000; monoclonal antibody; MP Biomedicals, Aurora, OH, USA). The secondary antibody used for pJAK2, JAK2 and RGS7 was goat anti-rabbit. Goat-anti mouse was used for actin protein. Prior to incubation with a second primary antibody, blots were stripped with Restore Western blot stripping buffer (Pierce) by incubating at 37 °C for 25 min. After incubation, blots were removed from stripping buffer, washed three times for 10 min each with TBS or PBS containing 0.1% Tween-20 (TBST or PBST) and blocked with 5% milk in TBST or PBST for 1 h at room temperature. Protein bands were analysed densitometrically using Scion Image software (Scion Corporation, Frederick, MD, USA). The grey scale density readings were calibrated using a transmission step-wedge standard. The integrated optical density (IOD) of each band was calculated as the sum of the optical densities of all the pixels within the area of the band outlined. The IOD for the film background was subtracted from the IOD for each band. Each sample was measured in triplicate. RGS7 protein was normalized to actin protein and phosphoproteins were normalized to the corresponding total protein levels. Protein levels from treated cells were normalized to vehicle-treated cells for each Western blot analysis.

**IP accumulation assay**

The assay was performed as previously described (Singh et al., 2007). Briefly, cells were seeded in 24-well plates at a density of 40 000 cells/well. Cells were treated with vehicle or different concentrations of olanzapine or clozapine for 24 h in serum-free DMEM media during the same 24-h period; cells were labelled with 0.5 μCi [3H]myoinositol/well. Following treatment and labelling, cells were washed four times with HBSS mix containing 20 mM LiCl$_2$ and 20 mM Hepes. More specifically, cell culture media containing olanzapine and [3H]myoinositol was aspirated completely followed by addition of washing buffer. The plate was shaken to completely dissolve any residual media left in the wells. Washing buffer was aspirated carefully to remove buffer without affecting cells attached to the plate. This step was repeated three more times for a total of four washes; overall, cells were washed thoroughly four separate times to remove any residual drug or radioactivity present on cells. Cells were then incubated with 500 μl of the same buffer at 37 °C for 15 min. Following the incubation, cells were challenged with 100 μM DOI for 30 min. We previously performed a concentration response experiment examining the effects of DOI on IP accumulation in A1A1v cells and found that the EC$_{50}$ is 1 μM DOI and the $E_{\text{max}}$ is 100 μM DOI (Shi et al., 2007). In all subsequent experiments, we have used the $E_{\text{max}}$ concentration of DOI for the IP assays in A1A1v cells. Although DOI is also an agonist for 5-HT$_{2C}$ receptors, we demonstrated that in A1A1v cells, IP accumulation stimulated with 100 μM DOI is blocked by the selective 5-HT$_{2A}$ receptor antagonist MDL100907.
(Shi et al., 2007). Moreover, we found that A1A1v cells do not express detectable amounts of 5-HT<sub>2</sub>C receptors (Dai et al., 2008).

Media was replaced with 10 mM ice-cold formic acid after the challenge treatment, and incubated on ice for 1 h. AG1-X8 (Bio-Rad Laboratories, Hercules, CA, USA) resin columns were prepared as follows: columns were washed once with 3 ml of 3 M ammonium formate/100 mM formic acid, twice with 5 ml of 10 mM formic acid/10 mM inositol. Once the columns were drained out completely, samples were loaded into the column and allowed to enter into the resin. Columns were then washed once with 5 ml of 10 mM formic acid/10 mM inositol, twice with 5 ml of 60 mM sodium formate/5 mM borax. After washing, samples were eluted with 5 ml of 1 M ammonium formate/100 mM formic acid into scintillation vials, 12 ml of scintillation cocktail was added into each vial, mixed thoroughly and counted in a scintillation counter.

**PLC assay**

Since preincubation with AG490 interferes with [3H]myoinositol incorporation into A1A1v cells, we used an alternative, ex-vivo method to isolate membranes from control and treated cells and incubated the membrane fraction with [3H]phosphatidylinositol. This method involves testing the enzymatic activity of PLC present in isolated membranes thereby avoiding any problems with incorporation of [3H]myoinositol in presence of AG490. To harvest cells, cell culture media was aspirated and then washed twice with ice-cold PBS to completely remove any residual media. The cells were scraped off the plate in Tris buffer [25 mM Heps-Tris, 1 mM EGTA (pH 7.4), containing protease inhibitor cocktail] and spun at 20 000 g for 20 min at 4 °C. The pellet was resuspended in Tris buffer and stored at −80 °C. The pellet was thawed on the day of the PLC assay and homogenized by hand with five up-and-down strokes with a glass-on-glass homogenizer and then centrifuged at 20 000 g for 20 min. The supernatant was discarded and the pellet was re-suspended in 50 mM Tris buffer with slow vortex to make a complete suspension. This suspension was then spun at 20 000 g for 10 min. The supernatant was discarded and the pellet was re-suspended in assay buffer [25 mM Tris (pH 7.4), 3 mM EGTA, and 10 mM LiCl] with slow vortex to make a complete suspension. The suspension was spun at 20 000 g for 10 min to collect a pellet. This step was repeated two more times to complete three washes of the membrane preparation before use for the PLC assay. Overall, both treated and control cells were washed multiple times before the membrane preparation from these cells was used for PLC assay. 5-HT- and GTP<sub>S</sub>-stimulated PLC activity in cell membranes were measured as described previously (Damjanoska et al., 2003; Wolf and Schutz, 1997). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). The membrane protein was diluted to an approximate concentration of 30 μg/100 μl with buffer containing 25 mM Hepes-Tris, 3 mM EGTA, 10 mM LiCl, 12 mM MgCl<sub>2</sub>, 1.44 mM sodium deoxycholate with 0.5 μM GTP<sub>S</sub> (a non-hydrolysable form of GTP), 100 mM free Ca<sup>2+</sup>, 1 mM unlabelled phosphatidylinositol, and 100 μM [3H]phosphatidylinositol (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). A concentration of 100 μM 5-HT or 1 μM of Bradykinin was used to stimulate PLC activity. 5-HT-stimulated PLC activity is a selective measure of 5-HT<sub>2</sub>A receptor function in A1A1v cells as previously demonstrated using selective antagonists (Shi et al., 2007). Bradykinin was used to investigate the selectivity of olanzapine-induced desensitization of 5-HT<sub>2</sub>A receptor signalling.

**RNA isolation and reverse transcription**

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

**Real-time PCR**

The GAPDH (sense: 5'-tgagctacttgccgcttccac-3'; antisense: 5'-ggcatgactggtgtatca-3') and RGS7 (sense: 5'-gaagatgagttgcaccga-3'; antisense: 5'-ggcttcaggtgctctcat-3') primer sets were synthesized by IDT Inc. (Coralville, IA, USA). PCR amplification was performed with 7500 Real-Time PCR System using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The PCR parameters used were a 10 min denaturation cycle at 95 °C, 40 cycles of amplification at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Real-time PCR was performed with
25 µl reaction mixture of cDNA, primers and SYBR Green master mix.

**RNA data analysis**

Comparative C_T (ΔΔC_T) method was used for analysis of all real-time PCR data. ΔC_T values were calculated by normalizing C_T values of RGS7 to GAPDH from vehicle- and antagonist-treated groups. The extent of the response is determined by 2^mean(ΔΔC_T), and the relative degree of response is calculated by 2^mean(ΔΔC_T). Results are expressed as fold change in RGS7 mRNA levels for clozapine-, MDL 100907- or olanzapine-treated cells with respect to vehicle-treated cells. Data presented are from four independent experiments performed in triplicate.

**Chromatin immunoprecipitation (ChIP) assay**

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

- **R1**: 5'-AATTTGGAGGCCTGGACC-3',
- **R2**: 5'-ATTATGGAGCGCTGGACC-3',
- **R3**: 5'-ATCCTTGGCACTGGACACC-3',
- **R4**: 5'-AGGTGGTTGCCACCTTGTCG-3',
- **R5**: 5'-ACATCTCAGGACGGAG-3',

A previously identified STAT3 binding region from the hepcidin gene was a positive control (F: 5'-GAGGTGTGACAACCTGTT-3', R: 5'-ACCGAG-TGACA GTCGTTT-3') (Wrighting and Andrews, 2006). Two microlitres of precipitated DNA was amplified using Taq polymerase (New England Biolabs, Ipswich, MA, USA). The conditions for PCR amplification were as follows: 40 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 45 s, and the amplimers were resolved on 1% agarose gels containing etidium bromide.

**Statistics**

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

**Results**

**IP accumulation assay**

Agonist-stimulated IP accumulation can be used to monitor desensitization of 5-HT_2A receptor signalling (Singh et al., 2007). Treatment with olanzapine for 24 h significantly decreased (F_3,8=49.83, p<0.0001) DOI (10^-4 M)-stimulated IP accumulation in a dose-dependent manner. Treatment with 30 nM, 300 nM or 3000 nM of olanzapine decreases the amount of IP by ~13% (p<0.05), 27% (p<0.01) or 51% (p<0.01), respectively, compared to vehicle (acetic acid)-treated cells (Figure 1a). A similar effect on IP accumulation was also observed when cells were treated with clozapine at a higher concentration range. We found that treatment with increasing concentrations of clozapine for 24 h significantly decreased (F_4,14=104.43, p<0.0001) DOI-stimulated IP accumulation in a dose-dependent manner compared to vehicle (DMSO)-treated cells (Figure 1b). A post-hoc analysis revealed a decrease in IP accumulation by 39% with 5 µM (p<0.01), 53% with 20 µM (p<0.01), 64% with 30 µM (p<0.01), and 80% with 40 µM (p<0.01) treatment. Subsequent experiments used 20 µM clozapine based on this dose resulting in a reduction of ~50% of the IP accumulation. These decreases suggest a desensitization of 5-HT_2A-mediated receptor signalling in A1A1v cells by olanzapine or clozapine.

**Clozapine and MDL 100907 treatment increased phosphorylation of JAK2 and pretreatment with AG490 abolished this effect**

We have shown that the 5-HT_2A receptor inverse agonist, olanzapine causes phosphorylation of JAK2 kinase (Singh et al., 2007). In order to investigate whether the effect is specific to olanzapine or is a general effect of atypical antipsychotics and more specifically 5-HT_2A receptor antagonists, we treated A1A1v cells for 24 h with either clozapine, MDL 100907 or drug vehicles. Membrane fractions prepared from...
vehicle-, clozapine (20 μM)-, and MDL 100907 (1 μM)-treated cells were analysed by Western blot with an anti-phospho-JAK2 antibody, then stripped and reprobed with an anti-JAK2 antibody (Figure 2a). Tyrosine phosphorylation of JAK2 was significantly increased ($F_{2,8}=39.57$, $p<0.001$). A post-hoc analysis revealed that pJAK levels were increased to 183 ± 13% of the control levels with MDL 100907-treated cells and 196 ± 18% of the control levels with clozapine-treated cells, whereas total JAK2 protein levels did not show any appreciable change. To investigate whether inhibition of the JAK-STAT signalling cascade could reverse the increase of phosphorylation of JAK2 observed in response to clozapine or MDL 100907 treatment, cells were pretreated for 1 h with 30 μM AG490, a JAK kinase inhibitor, prior to incubation with either clozapine or MDL 100907 for 24 h. As shown in Figure 2b, the clozapine- or MDL 100907-induced increase in phosphorylation of JAK2 was blocked by AG490 pretreatment. Two-way ANOVA indicates a significant main effect of AG490 pretreatment ($F_{1,12}=1469.36$, $p<0.001$), a significant main effect of clozapine or MDL 100907 incubation ($F_{2,12}=22.28$, $p<0.01$) on JAK2 phosphorylation and a significant interaction between these two factors ($F_{2,12}=35.75$, $p<0.01$). JAK2 phosphorylation is significantly greater in cells incubated with clozapine or MDL 100907 and pretreated with vehicle compared to the other four groups ($p<0.01$).

**Pretreatment with AG490 prevented clozapine- and MDL 100907-induced increases in RGS7 protein levels**

Next, we wanted to determine whether this increase in JAK2 phosphorylation with both clozapine and MDL 100907 is also accompanied by increases in RGS7 protein levels as previously observed with olanzapine. In order to monitor the changes in RGS7 protein levels, membrane fractions of vehicle-, clozapine-, or MDL 100907-treated cells were analysed by Western blot with anti-RGS7 antibody (Figure 3a). We found that RGS7 protein levels were significantly increased ($F_{2,8}=95.99$, $p<0.001$) by drug treatments. A post-hoc analysis revealed that RGS7 protein levels were increased to 176 ± 16% of the control levels with MDL 100907-treated cells and 194 ± 11% in clozapine-treated cells. Pretreatment with AG490 for 1 h inhibits the increase of RGS7 protein in response to clozapine or MDL 100907 stimulation (Figure 3b). Two-way ANOVA indicates a significant main effect of AG490 pretreatment ($F_{1,12}=20.05$, $p<0.05$), a significant main effect of clozapine or MDL 100907 incubation ($F_{2,12}=13.14$, $p<0.05$) on RGS7 and a significant interaction between these two factors ($F_{2,12}=12.06$, $p<0.05$). RGS7 is significantly increased in cells treated with clozapine or MDL 100907 and pretreated with vehicle compared to the other four groups ($p<0.05$).

**JAK2 phosphorylation preceded the increase in RGS7 protein in response to olanzapine**

We previously demonstrated that olanzapine causes phosphorylation of JAK2 kinase and STAT3, and
increases RGS7 protein, suggesting that olanzapine-induced activation of the JAK-STAT signalling cascade leads to increased expression of RGS7 protein (Singh et al., 2007). To further explore the underlying mechanism, we treated cells with olanzapine (300 nM) or vehicle (20% acetic acid) for 3, 6, 12 or 24 h. Lysates of membrane fractions were examined by Western blot with anti-phospho-JAK2, or anti-RGS7 antibody, then stripped and reprobed with anti-JAK2 and anti-actin antibodies. The bar graph represents quantification of phospho-JAK2 protein levels divided by JAK2 protein levels from five independent experiments (indicates significantly different from vehicle-pretreated and the same drug-treated cells at p < 0.01; * indicates significantly different from vehicle-pretreated and vehicle-treated cells at p < 0.01 as analysed by two-way ANOVA and Newman–Keuls post-hoc tests).

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

We have previously shown that the olanzapine-induced activation of the JAK2-STAT3 pathway is necessary for the increase in RGS7 protein levels; next we wanted to determine if activation of the JAK2-STAT3 pathway is necessary for olanzapine-induced desensitization of 5-HT_{2A} receptor signalling. However, AG490 interfered with the measurements of IP...
Figure 3. Pretreatment with AG490 prevented the clozapine- and MDL 100907-induced increase in RGS7 protein levels. (a) A1A1v cells were treated either with vehicle (DMSO) or 20 µM clozapine or 1 µM MDL 100907 for 24 h. Membrane fractions of cells were analysed by Western blot with an anti-RGS7 antibody, stripped and reprobed with an anti-actin antibody as a loading control. Bar graph represents quantification of RGS7 protein levels divided by actin protein levels from four independent experiments. RGS7 protein levels were significantly increased ($p < 0.05$) with clozapine and MDL 100907 compared to vehicle-treated cells (* indicates significantly different from vehicle-treated cells at $p < 0.05$). (b) Cells were pretreated with AG490 (30 µM) for 1 h before treatment with either MDL 100907 or clozapine for 24 h. Membrane fractions of cells were analysed as described above. Bar graph represents quantification of RGS7 protein levels divided by actin protein levels from three independent experiments (# and ## indicates significantly different from vehicle-pretreated and the same drug-treated cells at $p < 0.05$ and $p < 0.01$ respectively; * indicates significantly different from vehicle-pretreated and vehicle-treated cells at $p < 0.05$ as analysed by two-way ANOVA and Newman–Keuls post-hoc tests).

Figure 4. Time-course studies show JAK2 phosphorylation preceded the increase in RGS7 protein in response to olanzapine. (a) Cells were treated with olanzapine (300 nM) or vehicle (20% acetic acid) for 3, 6, 12 or 24 h. Membrane fractions of cells were analysed by Western blot with anti-phospho-JAK2, then stripped and reprobed with anti-JAK2 antibody. The bar graph represents quantification of phospho-JAK2 protein levels divided by JAK2 protein levels from three independent experiments (* indicates significantly different from vehicle-treated cells at the same time-point at $p < 0.01$; # and ## indicate significantly different from 3 h olanzapine-treated cells at $p < 0.05$ and $p < 0.01$ respectively; + indicates significantly different from 6 h olanzapine-treated cells at $p < 0.01$). (b) The same membrane was stripped and reprobed with anti-RGS7 and anti-actin antibody. Bar graph represents quantification of RGS7 protein levels divided by actin protein levels from three independent experiments (* indicates significantly different from vehicle-treated cells at the same time-point at $p < 0.01$; # indicates significantly different from 3 h olanzapine-treated cells at $p < 0.01$ as analysed by two-way ANOVA and Newman–Keuls post-hoc tests).
accumulation in vivo. Therefore, we decided to use an alternative ex-vivo method in which we isolated membranes from AG490-pretreated, control and olanzapine-treated cells and then incubated them with [3H]phosphatidylinositol. This method involves measuring the enzymatic activity of PLC present in isolated membranes thereby avoiding any interference of pre-incubation of AG490 on incorporated [3H]myoinositol. Olanzapine-induced changes in PLC activity were monitored in response to stimulation with 5-HT, bradykinin, or GTP\textsubscript{S}. Basal activity was 241.6 pmol/mg protein per min whereas 5-HT-stimulated PLC activity measurement was 2431.0 pmol/mg protein per min. 5-HT stimulation measures the ability of 5-HT\textsubscript{2A} receptors to stimulate PLC activity via \(G_{a_{q/11}}\) activation whereas GTP\textsubscript{S} directly binds to \(G_{a_{q/11}}\) to activate PLC. Bradykinin was used to investigate the specificity of olanzapine-induced desensitization response by examining the effects on another \(G_{a_{q/11}}\) linked receptor system. Olanzapine treatment significantly decreased (by 55\%, \(p < 0.01\)) PLC activity stimulated with 5-HT. AG490 alone had no effect on PLC activity (Figure 5a). However, pretreatment with AG490 significantly attenuated the olanzapine-induced decreases in PLC activity (\(p < 0.05\)) suggesting that the JAK-STAT pathway is necessary for the full olanzapine-induced desensitization of 5-HT\textsubscript{2A} receptor signalling. Two-way ANOVA indicates a main effect of olanzapine on PLC activity (\(F_{1,19} = 41.18, p < 0.001\)), a main effect of AG490 (\(F_{1,19} = 4.23, p < 0.05\)), but no significant interaction was observed between olanzapine and AG490 (\(F_{1,19} = 2.92, p < 0.391\)). In addition, olanzapine treatment had no effect on bradykinin-stimulated PLC activity confirming that olanzapine treatment specifically desensitizes 5-HT\textsubscript{2A} receptor signalling (Figure 5c).

**Figure 5.** A JAK inhibitor partly attenuated the olanzapine-induced decrease in PLC activity. A1A1v cells were pretreated for 1 h with 30 μM AG490 prior to treating with either vehicle (20% acetic acid) or 300 nM olanzapine for 24 h. (a) 5-HT-stimulated PLC activity was significantly (\(p < 0.01\)) reduced by olanzapine treatment compared to vehicle-treated control (\(**\) indicates significantly different at \(p < 0.01\)). AG490 alone did not have any effect on PLC activity as analysed by two-way ANOVA and Newman–Keuls post-hoc tests. However, in cells pretreated with AG490, the olanzapine-induced decrease in PLC activity was significantly attenuated (\(*\) indicates significantly different at \(p < 0.05\)). (b) GTP\textsubscript{S}-stimulated PLC activity was not altered either by the olanzapine or AG490 treatments. (c) Olanzapine treatment had no effect on bradykinin-stimulated PLC activity whereas 5-HT-stimulated PLC activity was significantly reduced suggesting olanzapine treatment selectively affect 5-HT\textsubscript{2A} receptor-mediated PLC activity as analysed by two-way ANOVA and Newman–Keuls post-hoc tests. Experiments were performed three independent times.

Olanzapine, clozapine and MDL 100907 increase RGS7 mRNA levels

To investigate whether the increase in RGS7 protein levels is associated with an increase in RGS7 mRNA...
levels, cells were treated in a similar fashion as in previous experiments for 24 h with vehicle, olanzapine, clozapine, or MDL 100907. Total RNA was isolated from each sample and reverse-transcribed with random hexamers to make cDNA. Using that cDNA, a real-time PCR analysis was performed with specific primers for RGS7 and GAPDH. The vehicle and drug-treated RGS7 mRNA levels were normalized to GAPDH. We found a statistically significant \( F_{1,15} = 43.43, p < 0.001 \) increase in RGS7 mRNA levels. A post-hoc analysis revealed that RGS7 mRNA levels were increased to 153 ± 11% with olanzapine (Figure 6a), 175 ± 14% with clozapine, and 144 ± 17% with MDL 100907 treatment (Figure 6b) over their respective vehicle-treated cells.

**STAT3 binds to the putative RGS7 promoter region**

To determine if STAT3 binds to potential STAT3 consensus sites in cells, we used a ChIP approach. A bioinformatic analysis of the 10 kb promoter region of rat RGS7 identified five potential STAT3-binding sites based on the consensus sequence TTCN_{2-4}GAA. Specific primers were designed that flank each of these consensus sites and were named site numbers 1–5 (Figure 7). A1A1v cells were treated with vehicle (20% acetic acid) or olanzapine (300 nm) for 24 h as previously described. Immunoprecipitates isolated from either vehicle- or olanzapine-treated cells were used to perform ChIP analysis. Of the five potential STAT3-binding sites identified, only site 2 tested positive in this analysis (Figure 7), while the other primers failed to result in binding to either the immunoprecipitated DNA or the input DNA. Moreover, treatment with olanzapine appeared to increase STAT3 binding at this site. However, this approach is only semi-quantitative so no attempt was made to quantify the increase in band intensity in the olanzapine-treated lanes. Site 2 begins 2.34 kb upstream of the RGS7 transcription start site. Negative control primers did not produce an amplimer using immunoprecipitated chromatin (data not shown). The primers specific for hepcidin, used as a positive control, also tested positive using immunoprecipitated STAT3. In contrast, when the beads alone or pre-immune IgG was used in the immunoprecipitation procedure, no amplimer was detected.

**Discussion**

Atypical antipsychotics have been previously reported to act as inverse agonists and to induce desensitization of 5-HT_{2A} receptor signalling (Egan et al., 1998, 2000; Herrick-Davis et al., 1998; Rauser et al., 2001). Internalization and down-regulation have been proposed as mechanisms of desensitization (Roth et al., 1995; Willins et al., 1998) however, subsequent studies provided evidence supporting both internalization- and down-regulation-dependent and independent desensitization of 5-HT_{2A} receptors by atypical antipsychotics (Hanley and Hensler, 2002; Kuoppamaki et al., 1995). It is likely that internalization and down-regulation can contribute to the desensitization...
process but whether they are necessary and sufficient for the full desensitization process is not known. In addition, studies were inconclusive regarding the role for transcriptional regulation of 5-HT$_2$A receptor down-regulation with antipsychotics (Gray and Roth, 2001). Both, a decrease in receptor mRNA in hippocampus, brainstem, and midbrain and no change in mRNA levels have been previously reported with atypical antipsychotics (Burnet et al., 1996; Doat-Meyerhoefer et al., 2005). Thus, transcriptional regulation of 5-HT$_2$A receptors by atypical antipsychotics does not appear to be responsible for down-regulation leading to the desensitization of 5-HT$_2$A receptor signalling.

Consistent with previous reports that atypical antipsychotics induce desensitization of 5-HT$_2$A receptor signalling (Gray and Roth, 2001) we find that olanzapine and clozapine induce desensitization of 5-HT$_2$A receptor-stimulated PLC activity in A1A1v cells. Olanzapine, clozapine, and MDL 100907 increase RGS7 mRNA and protein levels as well as the activation of the JAK-STAT pathway. We previously found that the increase in RGS7 protein expression in response to olanzapine treatment is dependent on JAK-STAT signalling. Olanzapine-induced decreases in PLC activity, monitored as an index of 5-HT$_2$A receptor responsiveness (i.e. desensitization), were significantly attenuated by pretreatment with a JAK2 kinase inhibitor. These data suggest that activation of the JAK-STAT pathway is necessary for the full atypical antipsychotic-induced desensitization of the 5-HT$_2$A receptor-stimulated PLC response, however, receptor down-regulation and internalization probably also contribute to the desensitization of 5-HT$_2$A receptor signalling.

In addition to 5-HT$_2$A receptors, atypical antipsychotics also have high affinity for other G protein-coupled receptors, e.g. other 5-HT receptors (e.g. 5-HT$_1$A, 5-HT$_2$C, 5-HT$_6$, and 5-HT$_7$) (Meltzer et al., 1989; Meltzer, 1999; Roth et al., 1992; Seeger et al., 1995), the dopamine D$_4$ receptor (Roth et al., 1995; Van Tol et al., 1991), all five muscarinic receptors (m$_1$–m$_5$) (Peroutka and Snyder, 1980; Zeng et al., 1997), and several adrenergic and histamine receptors (Peroutka and Snyder, 1980). MDL 100907 initially characterized as a selective antagonist of the 5-HT$_2$A receptor, has been used to delineate antipsychotic responses mediated specifically by the 5-HT$_2$A receptor (Bhagwagar et al., 2006; Olijslagers et al., 2005; Wolff and Leander, 2000) and desensitizes 5-HT$_2$A receptor signalling (Rauser et al., 2001). In the present study, we report that MDL 100907 stimulated activation of the JAK-STAT pathway and increased RGS7 protein and mRNA levels suggesting that antagonism of 5-HT$_2$A receptors is sufficient to induce these changes. Similar effects could be caused by antagonism of 5-HT$_2$A receptors by olanzapine and

Figure 7. STAT3 binds to the putative RGS7 promoter region at site 2. ChIP assays were performed as described in the Materials and Methods section with an anti-STAT3 antibody, non-immune IgG, or with beads alone. Primers that amplify the STAT3 binding site previously identified in the hepcidin gene were used as a positive control. Non-precipitated genomic DNA (input) was used as a positive control. Amplification was not observed in lanes loaded with samples immunoprecipitated with beads alone, i.e. without the STAT3 antibody, and non-specific IgG in place of the STAT3 antibody confirming specificity of the reaction with the STAT3 antibody.
clozapine, although the binding of these atypical antipsychotics to other receptors could also contribute to responses and cannot be ruled out.

RGS proteins reduce G-protein-mediated signalling by acting as guanosine triphosphatase (GTPase)-accelerating proteins (GAPs) for Gα subunits and by blocking the interaction of Gα subunits with effectors (Dohlman and Thorner, 1997; Hollinger and Hepler, 2002). Expression of RGS7 protein in rat frontal cortex is well documented (Krumins et al., 2004; Zhang and Simonds, 2000) and decreased 5-HT2A receptor signalling via direct interaction of RGS7 protein with Gαq has been characterized in different systems (DiBello et al., 1998; Ghavami et al., 2004). Furthermore, an increase in RGS7 protein following both olanzapine and clozapine treatment would increase the termination rate of 5-HT2A receptor-Gαq/11 protein signalling by more rapidly hydrolysing GTP, and could thereby produce or contribute to the desensitization response. However, since GTPγS is a non-hydrolysable GTP analogue, PLC activity stimulated by GTPγS alone would not be expected to be affected by RGS proteins. Indeed, as shown in Figure 5b, GTPγS-stimulated PLC activity was not altered by the increase in RGS7 protein that occurs with olanzapine treatment. Therefore, the differential effects of olanzapine on receptor vs. G-protein activation of PLC activity are consistent with an increase in RGS7 protein either acting as a GAP for endogenous GTP induced by 5-HT to bind to Gαq/11 or by possibly blocking interaction of Gαq/11 with 5-HT2A receptors. Previous studies have demonstrated that RGS proteins can block the interaction of Gα subunits with effectors (Hollinger and Hepler, 2002; Ross and Wilkie, 2000) and so RGS7 could conceivably block the interaction of Gαq/11 with receptors. Further studies are needed to determine the mechanisms by which RGS7 is affecting the system.

Numerous studies have reported a significant decrease in RGS4 expression in the prefrontal cortex of schizophrenia subjects (Bowden et al., 2007; Erdely et al., 2006; Gu et al., 2007; Mirnics et al., 2001). Expression of RGS4 and RGS7 have been previously noted to be independent (Krumins et al., 2004). Like RGS7 proteins, RGS4 also regulates 5-HT2A receptor signalling. Atypical antipsychotic-induced increases in RGS7 levels observed in our studies might restore the 5-HT2A receptor signalling duration to physiological levels by substituting for the diminished RGS4 protein in schizophrenia patients.

Atypical antipsychotics could increase RGS7 levels by either increased stability of RGS7 protein or by increased transcription of RGS7 mRNA. RGS7 binding to Gβ5 is reported to increase the stability of each protein (Chen et al., 2003; Krumins et al., 2004). In addition, RGS7 phosphorylation and subsequent binding to 14-3-3 sequesters RGS7 in the cytoplasm (Burchett, 2003). Therefore, an increase in phosphorylation of RGS7 or increased expression of 14-3-3 or Gβ5 could increase the levels of RGS7 in the cytoplasm. Our real-time PCR data suggest that the increase in RGS7 levels by olanzapine, clozapine and MDL 100907 could be directly mediated by an increase in RGS7 mRNA via activation of the JAK-STAT pathway. STAT3 regulates a variety of biological processes, functioning at both transcriptional and non-transcriptional levels to influence cell growth, survival and metabolism (Inghirami et al., 2005; Leeman et al., 2006). From a genomic sequence analysis of rat RGS7, we have identified multiple sets of the STAT3 consensus binding element, TTCN4,GAA (Deckar et al., 1997; Ehret et al., 2001), suggesting that STAT3 could be a possible transcription factor for RGS7. The promoter region of RGS7 is not yet identified, however, it is usually present upstream of the transcription start site with our identified STAT3 binding site 2.34 kb upstream from the transcription initiation site. Taken together, these results are consistent with our hypothesis that activation of the JAK-STAT pathway by atypical antipsychotics and the subsequent increase in RGS7 expression is an underlying mechanism for desensitization of 5-HT2A receptor signalling.

In our previous studies (Muma et al., 2007; Singh et al., 2007) we have reported olanzapine-induced activation of the JAK-STAT pathway. In the present study we demonstrate that activation of the JAK-STAT pathway is necessary for full desensitization of 5-HT2A receptor signalling by atypical antipsychotics in A1A1v cells. While the precise mechanism of the JAK-STAT activation by atypical antipsychotics is not determined, it has been reported that 5-HT2A 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 5-HT2A receptors (Banes et al., 2005). Furthermore, atypical antipsychotics have also been reported to activate other signalling cascades for example, activation of ERK1/2 pathways in the rat frontal cortex.
(Fumagalli et al., 2006), Akt/PKB and P38 pathways in PC12 cells (Lu et al., 2004), and GSK3α/β in the rat frontal cortex (Kang et al., 2004; Roh et al., 2007). Although atypical antipsychotics have been extensively characterized as inverse agonist/antagonists of 5-HT₂A receptors, activation of the JAK-STAT pathway in our studies clearly indicates that besides being an antagonist, atypical antipsychotics are also an agonist for the JAK-STAT pathway. Our studies demonstrate that agonist activity as demonstrated by activation of the JAK-STAT pathway and antagonist effects at the PLC enzyme occur simultaneously. Previous studies have demonstrated selective agonism, where one agonist stimulates one pathway preferentially over another (Kenakin, 2007). Our studies extend the diversity of signalling by a single receptor suggesting that a ligand like MDL 100907 can be an agonist for one 5-HT₂A receptor-mediated pathway, JAK-STAT, and simultaneously an antagonist at the Gα₅/₁₁-PLC pathway.

Overall, our data suggest that desensitization of 5-HT₂A receptor-stimulated PLC activity by olanzapine, clozapine and MDL 100907 requires activation of the JAK-STAT pathway. In addition, activation of the JAK-STAT pathway and increases in RGS7 expression by transcriptional activity of STAT3 on the putative promoter site of RGS7.

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Statement of Interest

None.

References


