Agonist Induced-Phosphorylation of G_{\alpha11} Protein Reduces Coupling to 5-HT_{2A} Receptors

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Received March 19, 2007; accepted July 20, 2007

ABSTRACT

We previously demonstrated that 24-h treatment with (-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI) causes phosphorylation of G_{\alpha11} protein at serine 154 and that this phosphorylation causes desensitization of serotonin (5-HT) 2A receptor signaling in A1A1v cells (Shi et al., 2007). We now report that treatment of A1A1v cells with DOI for 24 h produces a greater reduction in the B_{max} of [\textsuperscript{125}I](\pm)-DOI-labeled high-affinity binding sites (46%) than the reduction of [\textsuperscript{3}H]ketanserin binding sites (25%). Although the K_{D} values are not altered, there is a smaller amount of GTP\_S [guanosine 5’-3-O-(thio)triphosphate]-sensitive [\textsuperscript{125}I](\pm)-DOI binding in DOI-treated cells. These results suggest that DOI treatment causes down-regulation of 5-HT_{2A} receptors and reductions in G protein-coupled 5-HT_{2A} receptors. In contrast, in cells transfected with the phosphorylation state mimic G_{\alpha11},S154D, GTP\_S-sensitive [\textsuperscript{125}I](\pm)-DOI binding was decreased by 48%; however, there was no significant difference in the K_{D} and B_{max} values of [\textsuperscript{125}I](\pm)-DOI-labeled receptors. The receptor binding experiments suggest that phosphorylation of G_{\alpha11} on serine 154 reduces coupling of 5-HT_{2A} receptors, whereas DOI causes down-regulation of 5-HT_{2A} receptors in addition to the phosphorylation-induced uncoupling of G_{\alpha11} to 5-HT_{2A} receptors.

To determine whether DOI increases phosphorylation of G_{\alpha11} protein in vivo, rats were treated with 1 mg/kg/day DOI or saline for 1 to 7 days. Seven days of DOI treatment significantly decreased phospholipase C activity stimulated by an E_{max} concentration of 5-HT by 40% and increased phosphorylation of G_{\alpha11} proteins by 51% in the frontal cortex. These data suggest that DOI causes phosphorylation of G_{\alpha11} in vivo and could thereby contribute to the desensitization of 5-HT_{2A} receptors.

Serotonin (5-HT) 2A receptors play important roles in the central nervous system (Roth et al., 1986; Hoyer et al., 1994). Desensitization of 5-HT_{2A} receptor signaling may underlie the mechanism of action of several drug treatments for neuropsychiatric disorders (Dean and Hayes, 1996). For example, several antipsychotic drugs, such as olanzapine, desensitize both 5-HT_{2A} and 5-HT_{2C} receptors (Kuoppamaa et al., 1995; Leysen et al., 1993; Roth and Ciaranello, 1991; Schmidt et al., 1995). Mechanisms leading to and resulting from the desensitization of 5-HT_{2A} receptors may explain the 2-week delay in full symptomatic improvement seen with these antipsychotic drugs. However, the molecular mechanisms that underlie the desensitization of 5-HT_{2A} receptor signaling are not well understood.

Previous studies suggested that three possible mechanisms might be involved in the desensitization of 5-HT_{2A} receptors: receptor uncoupling from G proteins, internalization (sequestration of the receptor away from the cell surface) (Bhattacharyya et al., 2002; Hanley and Hensler, 2002), or down-regulation (reduced ligand-bound receptor). For example, some in vivo and in vitro studies have demonstrated that agonist exposure resulted in the desensitization and down-regulation 5-HT_{2A} receptors (Anji et al., 2000; Buckholtz et al., 1988; McKenna et al., 1989; Valdez et al., 2002). However, other studies showed that agonists may cause desensitization without down-regulation or desensitization in the presence of increased densities of 5-HT_{2A} receptors (Akiyoshi

ABBRVIATIONS: 5-HT, serotonin; DOI, (-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; IOD, integrated optical density; PLC, phospholipase C; ANOVA, analysis of variance; E-64, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutyramide; GTP\_S, guanosine 5’-3-O-(thio)triphosphate; MDL 100,907, 4-piperidinemethanol, 1-[2-[4-fluorophenyl]ethyl]-\alpha-(2,3-dimethoxyphenyl)-, (\alphaR).
et al., 1993; Grotewiel and Sanders-Bush, 1994; Roth et al., 1995). The reduction of G-protein coupling to 5-HT$_{2A}$ receptors has been suggested by a greater reduction in the $B_{max}$ of agonist DOI ($-1$-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane HCl]-labeled high-affinity receptors than the reduction in $B_{max}$ of antagonist ketanserin-labeled 5-HT$_{2A}$ receptors after chronic agonist treatment (McKenna et al., 1989). These studies suggest that multiple mechanisms may be responsible for the desensitization of 5-HT$_{2A}$ receptors, but with the specific mechanism(s) involved, dependent on the specific cellular milieu in which the receptors are expressed (Roth et al., 1998). These findings further suggest that, although studies using cell culture models are informative, it is important to determine whether molecular mechanisms revealed in cell culture models reflect the nature of mammalian brain using complementary in vivo studies.

Our previous data in an embryonic rat cortical cell line, A1A1v cells, demonstrated that phosphorylation of G$_{o/q/11}$ protein on serine 154 by protein kinase C and calcium-calmodulin dependent kinase II contributes to agonist-induced desensitization of 5-HT$_{2A}$ receptor signaling (Shi et al., 2007). Exposure of A1A1v cells to DOI for 24 h increased phosphorylation of G$_{o/q/11}$ protein. Using site-directed mutagenesis, we found that mutation of serine 154 to alanine on G$_{o/q/11}$ protein reduced the desensitization of 5-HT$_{2A}$ receptor signaling and prevented the increase in phosphorylation of G$_{o/q/11}$ protein caused by DOI. Mutation of G$_{o/q/11}$ protein serine 154 to aspartic acid, a phosphorylation mimic, directly reduced 5-HT$_{2A}$ receptor signaling. In contrast, mutation of G$_{o/q/11}$ to alanine had no effect on agonist-induced desensitization of 5-HT$_{2A}$ receptor signaling.

As described above, it is important to determine whether the mechanisms revealed in cell culture models reflect the nature of the response in vivo; therefore, we examined the phosphorylation of G$_{o/q/11}$ protein in rat brain. Now we report the outcome of a time-course study to determine the impact of DOI on phosphorylation of G$_{o/q/11}$ protein and desensitization of 5-HT$_{2A}$ receptor signaling in rat frontal cortex. In rat frontal cortex, G$_{o/q/11}$ protein mRNA is more abundant than G$_{o/q/11}$ protein mRNA (Tanaka et al., 2000), suggesting that increased phosphorylation of G$_{o/q/11}$ protein would affect 5-HT$_{2A}$ receptor signaling in this brain region. Furthermore, we examined the effects of another drug that alters 5-HT$_{2A}$ receptor signaling on phosphorylation of G$_{o/q/11}$ protein, a serotonin reuptake inhibitor, fluoxetine, which increases 5-HT$_{2A}$ receptor signaling.

We further hypothesize that phosphorylation of G$_{o/q/11}$ protein reduces 5-HT$_{2A}$ receptor signaling by altering the interactions of G$_{o/q/11}$ protein with other proteins such as the 5-HT$_{2A}$ receptors. This hypothesis is consistent with our previous studies, which measured phospholipase C (PLC) activity stimulated by a EC$_{50}$ dose of 5-HT or GTP$_{S}$ [guanosine 5'-3-O-(thio)triphosphate], and suggested that an alteration at the receptor or an alteration in coupling of 5-HT$_{2A}$ receptors to G$_{o/q/11}$ protein occurs with agonist treatment (Damjanoska et al., 2004). To determine the impact of phosphorylation of G$_{o/q/11}$ protein and agonist treatment on 5-HT$_{2A}$ receptor coupling, we examined the density ($B_{max}$) and receptor affinity ($K_D$) of 5-HT$_{2A}$ receptors by using radioligand binding assays with $^{[125]}I(-)-$DOI, which labels high-affinity state of the 5-HT$_{2A}$ receptor, and $[^3]H$ketanserin, which labels total 5-HT$_{2A}$ receptor density. Most importantly, we examined coupling of G proteins to 5-HT$_{2A}$ receptors by measuring the inhibitory effects of GTP$_{YS}$ (tetrasodium salt solution) on $^{[125]}I(-)-$DOI binding.

### Materials and Methods

**Phosphorylation of G$_{o/q/11}$ Uncouples 5-HT$_{2A}$ Receptors**

Dulbecco’s modified Eagle’s medium and Lipofectamine Plus were supplied by Invitrogen (Carlsbad, CA). DOI and GTP$_{S}$S were purchased from Sigma-Aldrich (St. Louis, MO). F$[^3]$F[Phosphatidylinositol, $[^3]$H]ketanserin (specific activity, 76.5 Ci/mmol), and $^{[125]}I(-)-$DOI (specific activity, 2200 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

**Cell Culture and Transfections.** A1A1v neuronal cells endogenously express the 5-HT$_{2A}$ receptor signaling system and were kindly provided by Dr. William Clarke and Kelly Berg (University of Texas Health Science Center, San Antonio, TX). The A1A1v cells were grown in poly-L-ornithine-coated plates in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO$_2$. Serum was heat-inactivated and charcoal-treated to remove monomamines. Cells were treated with either 100 nM DOI or vehicle (Hanks’ balanced buffer) for 24 h before harvesting for the receptor-binding assay. Cells were transiently transfected with either wild-type G$_{o/q/11}$ or mutant G$_{o/q/11}$S154D using Lipofectamine Plus (Invitrogen) according to the manufacturer’s recommendations. A total of 4 μg/plate DNA was used in each transfection. After transfection (48 h), cells were harvested for radioligand binding assays.

**Cell Homogenates.** To harvest cells, culture plates were washed twice with ice-cold phosphate-buffered saline. We then added 50 mM Tris, pH 7.4, containing protease inhibitor cocktail (containing 104 μM 4-2-aminoethylbenzenesulfonic acid, 0.08 μM aprotinin, 2 μM leupeptin, 4 μM bestatin, 1.5 μM pepstatin A, and 1.4 μM E-64, pH 7.4) from Sigma-Aldrich. Cells were harvested by scraping cells off of the surface of the plate. Cells were then centrifuged at 30,000 g for 20 min at 4°C. The pellet was resuspended in 50 mM Tris buffer and stored at −80°C. The homogenates were thawed on the day of the binding assay and homogenized by hand with five up-and-down strokes with a glass/glass homogenizer and then centrifuged at 30,000g for 20 min. After centrifugation, the pellet was resuspended in 50 mM Tris buffer and incubated at 37°C for 15 min and centrifuged at 30,000g for 20 min. The resultant pellet was washed once and resuspended in binding assay buffer (50 mM Tris pH 7.4, 0.5 mM EDTA, and 10 mM MgSO$_4$). The final protein concentration was determined by using a bichinonic acid protein assay kit (Pierce Chemical, Rockford, IL).

**$^{[125]}I(-)-$DOI Saturation Analysis.** To determine changes in the density and affinity of high-affinity 5-HT$_{2A}$ receptors, increasing concentrations of $^{[125]}I(-)-$DOI (0.5–4.0 nM) were incubated with the cell homogenates (−100 μg/tube protein) for 1.5 h at room temperature in the same assay buffer as described above. The concentration range of $^{[125]}I(-)-$DOI was based on the $K_D$ in our previous study (Li et al., 1997). Nonspecific binding was defined in the presence of 100 nM MDL 100,907, a selective 5-HT$_{2A}$ receptor antagonist. GTP$_{S}$ binding experiments were performed using a concentration (−0.4 nM) of $^{[125]}I(-)-$DOI below its $K_D$. The reaction was terminated by rapid filtration over Whatman GF/C glass fiber filters that had been presoaked with 1.0% polyethylenimine. The filters were then washed with 15 ml of ice-cold 50 mM Tris buffer, pH 7.4. The amount of $^{[125]}I(-)-$DOI on the filters was determined by using a Micromedic 4/200 Plus γ-counter. All radioligand receptor binding assays were performed in triplicate in a final volume of 0.5 ml. Specific binding was defined as the total binding minus nonspecific binding. Determination of $B_{max}$ and $K_D$ values by either computer-assisted analyses of saturation data (Prism; GraphPad Software, Inc., San Diego, CA) or linear transformation of saturation data via Scatchard plots gave comparable results.
[3H]Ketanserin Saturation Analysis. To measure the total 5-HT2A receptor density (Bmax) and receptor affinity (Kd), [3H]ketanserin radioligand binding assays were performed. In saturation experiments, a range of [3H]ketanserin concentrations (1.0–30.0 nM) was incubated with cell homogenates containing ~100 μg/tube protein for 1 h at room temperature in assay buffer. Prazosin (30 nM) was included in all assays to preclude binding to α1 receptors. Non-specific binding was defined in the presence of 1 μM spiperone. The reactions were terminated by filtration and washing as described above. The amount of [3H]ketanserin on the filters was counted using a Beckman LS 6500 scintillation counter. Determination of Bmax and Kd values by either computer-assisted analyses of saturation data (Prism; GraphPad Software, Inc.) or by linear transformation of saturation data via Scatchard plots gave comparable results.

Animals. Male Sprague-Dawley rats (225–275 g; Harlan Laboratories, 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. Seven to 11 rats were used per experimental group. All procedures were conducted in accordance with the National Institutes of Health (Institute of Laboratory Animal Resources, 1996) as approved by the Loyola University Institutional Animal Care and Use Committee.

Animal Treatment Procedures. The rats were handled during the treatment periods to acclimate them to human contact and to minimize stress. Rats were randomly assigned to the various experimental groups, cage mates in the same experimental groups. The body weight of each rat was recorded every other day.

Sustained Agonist (DOI) Treatment. Rats received daily injections of DOI dissolved in 0.9% saline (1 mg/kg i.p.) for 1, 4, or 7 days or 0.9% saline (1 ml/kg i.p.) for 7 days. Rats receiving DOI injections for 1 or 4 days were given injections of 0.9% saline (1 ml/kg i.p.) on the days prior to the commencement of DOI treatment. Thus, every group received injections for a total of 7 days, which allowed us to control for any potential injection effects. Twenty-four hours after the last DOI treatment, the rats were decapitated. Brains were quickly removed, frozen on dry ice, and stored at ~80°C for biochemical and molecular analyses.

Chronic Fluoxetine Treatment. Rats were injected with fluoxetine (10 mg/kg/day i.p.), dissolved in 0.9% saline or 0.9% saline (2 ml/kg i.p.) for 21 days. Eighteen hours after the last injection, the rats were decapitated. The brains were quickly removed, frozen on dry ice, and stored at ~80°C.

PLC Assay. 5-HT- and GTPγS-stimulated PLC activity in frontal cortex was conducted as described previously (Damjanoska et al., 2003, 2004; Wolf and Schutz, 1997). In brief, membrane fractions were prepared from rat frontal cortex. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical). The membrane protein was diluted to a concentration of 30 μg/100 μl with a buffer containing 25 mM HEPES-Tris, 3 mM EGTA, 10 mM LiCl, 12 mM MgCl2, 1.44 mM sodium deoxycholate with 1 μM GTPγS (a nonhydrolyzable form of GTP), 300 mM free Ca2+, 1 mM unlabeled phosphatidylinositol, and 100 μM [3H]phosphatidylinositol (PerkinElmer Life and Analytical Sciences). Two concentrations of 5-HT were used to stimulate PLC activity: 0.3 μM (EC50) and 10.0 μM (Emax) (Wolf and Schutz, 1997). This extends our previous study, which used only a single EC50 dose of 5-HT to measure the desensitization response to repeated DOI treatments (Damjanoska et al., 2004). 5-HT-stimulated PLC activity in the frontal cortex is a selective measure of 5-HT2A receptor function as previously demonstrated using selective antagonists (Wolf and Schutz, 1997).

Analysis of Phosphorylation of Gαq/11 Proteins. Frontal cortex tissues from rats treated with DOI and saline were homogenized in a 50 mM Tris buffer, pH 7.7, containing 150 mM NaCl, 10% sucrose, and 0.5 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 2 mM activated sodium orthovanadate, and 1:1000 protease inhibitor cocktail (Sigma-Aldrich). The homogenate was then centrifuged for 60 min at 20,000g. The pellet was resuspended by sonication in a 20 mM Tris buffer, pH 8, containing 1 mM EDTA, 100 mM NaCl, and 1% sodium cholate. The resuspended pellet was agitated at 4°C for 60 min and was then centrifuged for 60 min at 100,000g. The supernatant was saved from each sample and stored at ~80°C before use in the immunoprecipitation assay. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Chemical).

Immunoprecipitation of Gαq/11 proteins was performed as described previously (Shi et al., 2007) using a Gαq/11 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The immunoprecipitated Gαq/11 proteins were resolved by loading 7 μl of supernatant from each sample onto a SDS-polyacrylamide gel containing 0.1% SDS, 8% acrylamide/bisacrylamide (30:0.2), 4.6 M urea, and 375 mM Tris, pH 8.7. Gels loaded with samples from the DOI experiment were designed to contain three samples from the saline-treated control group and three samples from each DOI treatment group. Gels loaded with samples from the fluoxetine experiment contained six samples from the 21-day fluoxetine treatment group and six samples of the 21-day saline treatment group. The proteins were electrophoretically transferred from the gels onto nitrocellulose or polyvinylidene difluoride membranes, and phosphorylated proteins were detected with a phospho-Ser/Thr/Tyr antibody (1:200; Spring Bioscience, Fremont, CA) as described previously (Shi et al., 2007) or with the Pro-Q Diamond Phosphoprotein Stain as per the manufacturer’s directions (Invitrogen). Levels of Gαq/11 proteins were examined to verify equal loading of protein in each lane (using Gαq/11 antibody, 1:500; and a horseradish peroxidase-labeled, anti-rabbit antibody, 1:100,000; Santa Cruz Biotechnology Inc.).

Figs were analyzed densitometrically using the Scion Image program (Scion Corp., Frederick, MD). For normalization, the integrated optical density (IOD) of phosphorylated Gαq/11 protein bands on each blot were divided by the mean IOD of saline-treated animals and by the IOD of the respective Gαq/11 protein bands (independent of their phosphorylation state).

A total of eight samples per group were analyzed for the DOI experiment. For each animal, immunoprecipitations were performed in triplicate, and the Western blot analysis of each immunoprecipitated sample was performed at least twice. The data presented for the DOI treatment are the means of all three immunoprecipitations. For the fluoxetine experiment, a total of six samples per group were analyzed. Immunoprecipitations for the fluoxetine treatment paradigm were performed twice, and the Western blot analysis of each immunoprecipitated sample was performed at least twice. The data are the means of six samples in one representative assay.

Statistical Analyses. All data are presented as group means ± S.E.M. The Kd and Bmax data were analyzed using Student t tests. The GTPγS-sensitive binding data, 5-HT-stimulated PLC activity, and 5-HT to GTPγS ratio data were analyzed using a two-way ANOVA followed by a Newman-Keuls post hoc analysis. GTPγS-stimulated PLC activity was analyzed using one-way ANOVA. Because of nonhomogeneity of variance assessed using Bartlett’s Chi square (p < 0.05), data from the immunoprecipitation assays for the DOI-treatment paradigm were analyzed using nonparametric Kruskal-Wallis one-way ANOVA. This was followed by a Newman-Keuls post hoc analysis. The immunoprecipitation assays of the fluoxetine-treatment paradigm, comparing fluoxetine with saline treatment, were analyzed using a Student’s t test. GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD) was used for all statistical analyses. A probability level of p < 0.05 was considered to be statistically significant for all statistical tests.

Results

Effect of DOI Treatment on 5-HT2A Receptors. To determine whether there is a change in 5-HT2A receptors in A1A1v cells with a 24-h DOI treatment, saturation assays
(Fig. 1) were performed using \([^{125}I]\) (±)-DOI (which labels the high-affinity state of 5-HT\(_{2A}\) receptors) and \([^{3}H]\) ketanserin (which labels total 5-HT\(_{2A}\) receptor density). As shown in Table 1, the density of \([^{125}I]\) (±)-DOI labeled 5-HT\(_{2A}\) receptors in the high affinity state was significantly \((p < 0.001)\) lower in cells treated with DOI compared with vehicle-treated cells (Table 1). Cells treated with DOI showed 46.5% reduction in high-affinity 5-HT\(_{2A}\) receptors. The total density of \([^{3}H]\) ketanserin-labeled 5-HT\(_{2A}\) receptors also was significantly \((p < 0.01)\) lower in cells treated with DOI than in cells treated with vehicle (Table 1). A 24-h DOI treatment resulted in a 24.8% reduction in the total density of 5-HT\(_{2A}\) receptor sites. There were no significant changes in the affinity of either \([^{125}I]\) (±)-DOI or \([^{3}H]\) ketanserin for the receptor after 24 h of DOI treatment.

To further determine whether 24-h DOI-induced desensitization of 5-HT\(_{2A}\) receptors is a result of impaired capacity of the 5-HT\(_{2A}\) receptor to couple to G proteins, GTPγS-sensitive \([^{125}I]\) (±)-DOI binding was performed at a single concentration (0.4 nM). As shown in Fig. 2, treatment of cells with DOI for 24 h reduced the amount of \([^{125}I]\) (±)-DOI binding by 85.8% compared with vehicle-treated cells \(F(1,11) = 18.11, p < 0.001\). GTPγS (20 μM) significantly reduced the specific binding of \([^{125}I]\) (±)-DOI in vehicle-treated cells by 73.6% \(F(1,11) = 16.25, p < 0.01\). In cells treated with DOI for 24 h, GTPγS did not significantly reduce \([^{125}I]\) (±)-DOI binding. These data suggest that 24-h DOI treatment reduced the number of 5-HT\(_{2A}\) receptors coupled to G proteins rather than altering binding to a high-affinity non-G protein-coupled conformational state of the receptor.

**Effect of Expression of G\(_{11\text{S154D}}\) on \([^{125}I]\) (±)-DOI-Labeled High-Affinity State of the 5-HT\(_{2A}\) Receptor in A1A1v Cells.** The results from our previous study (Shi et al., 2007) suggested that phosphorylation at G\(_{11}\) serine 154 contributes to DOI-induced desensitization of 5-HT\(_{2A}\) receptor signaling. Here we sought to determine whether phosphorylation at G\(_{11}\) serine 154 residue decreased the coupling of G proteins with 5-HT\(_{2A}\) receptor. \([^{125}I]\) (±)-DOI-labeled density of 5-HT\(_{2A}\) receptors was examined in A1A1v cells transfected with either the phosphorylation state mimic G\(_{11\text{S154D}}\) or wild-type G\(_{11}\). The transfection efficiency was approximately 40 to 60%. As shown in Table 2, no significant difference in K\(\text{D}\) or the density of 5-HT\(_{2A}\) receptors was observed between cells transfected with wild-type G\(_{11}\) or the phosphorylation state mimic. GTPγS-sensitive \([^{125}I]\) (±)-DOI binding was then determined at the K\(\text{D}\) concentration (0.4 nM). No significant difference in the density of 5-HT\(_{2A}\) receptors was observed between cells transfected with wild-type G\(_{11}\), or phosphorylation state mimic at the K\(\text{D}\) concentration. However, in cells transfected with the phosphorylation state mimic G\(_{11\text{S154D}}\), GTPγS-sensitive binding was reduced to 47.6 ± 9.6% GTPγS-sensitive binding in cells transfected with wild-type G\(_{11}\) (Fig. 3). These data suggest less 5-HT\(_{2A}\)-G protein coupling with the G\(_{11\text{S154D}}\) phosphorylation mimic.

**Effect of in Vivo DOI Treatment on 5-HT-Stimulated PLC Activity.** As shown in Fig. 4A, 5-HT-stimulated PLC activity in the frontal cortex in a concentration-dependent manner. Pretreatment with daily injections of DOI (1 mg/kg) significantly decreased 10 μM 5-HT-stimulated PLC activity in the frontal cortex of rats \(F(2,42) = 11.1, p = 0.0001\). With the 10 μM concentration, 5-HT-stimulated PLC activity was decreased by 32% after 4 days of DOI treatment \((p < 0.01)\) and by 40% after 7 days \((p < 0.01)\) of DOI treatment (Fig. 4A). DOI treatment reduced the PLC response to 0.3 μM 5-HT by 26% at 4 days and 39% at 7 days, but this difference did not reach statistical significance (Fig. 4A). A two-way ANOVA revealed a significant effect of 5-HT \([^{2}F(1,42) = 19.3, p < 0.0001]\) and a significant effect of DOI \([^{2}F(2,41) = 4.46, p < 0.05]\), but there was no significant interaction between 5-HT and DOI treatment \([^{2}F(2,42) = 1.1, p > 0.05]\). As shown in Fig. 4B, DOI treatment for either 4 or 7 days did not significantly affect GTPγS-stimulated PLC activity in the frontal cortex \([^{2}F(2,21) = 0.6, p = 0.56]\). Although the GTPγS-stimulated PLC activity was not significantly altered, we examined the ratio of 5-HT- to GTPγS-stimulated PLC activity to determine whether variations in the GTPγS-stimulated PLC activity for individual animals would influence 5-HT-stimulated effects. As shown in Fig. 4C, DOI treatment decreased the ratio of 10 μM 5-HT- to GTPγS-stimulated PLC activity \([^{2}F(2,41) = 4.46, p = 0.02]\) by 30% after 4 days \((p < 0.05)\) and by 39% after 7 days \((p < 0.05)\), similar to that seen by 10 μM 5-HT stimulation directly (Fig. 4A). With 0.3 μM 5-HT stimulation, the DOI-induced reductions in the ratio on 5-HT to GTPγS-stimulated PLC activity were not statistically significant (Fig. 4C).
Effect of DOI treatment on the density of 5-HT\textsubscript{2A} receptors in A1A1v cells

Cells were treated with either vehicle or DOI (100 nM) for 24 h. \textsuperscript{[\textsuperscript{125}I]}\textsuperscript{z} DOI and \textsuperscript{[\textsuperscript{3H}]ketanserin saturation assays demonstrated reductions in the \(B_{\text{max}}\) of DOI-treated cells. Data represent the mean ± S.E.M. from three independent experiments. ** indicates a significant difference from vehicle-treated cells at \(P < 0.001\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(K_D) (nM)</th>
<th>(B_{\text{max}}) (fmol/mg)</th>
<th>(K_D) (nM)</th>
<th>(B_{\text{max}}) (fmol/mg)</th>
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<td>Vehicle</td>
<td>0.70 ± 0.02</td>
<td>10.69 ± 0.22</td>
<td>2.92 ± 0.02</td>
<td>130.48 ± 1.35</td>
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<tr>
<td>DOI</td>
<td>0.55 ± 0.07</td>
<td>5.72 ± 0.24**</td>
<td>2.79 ± 0.16</td>
<td>98.18 ± 2.57**</td>
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% Reduction
(−46.5)

Effect of DOI treatment on phosphorylation of \(G_{11}\) proteins in the frontal cortex (Student’s \(t\) test, \(p < 0.05\)).

\(G_{11}\) or \(G_{11}, S154D\) (4 \(\mu\)g/plate) for 48 h. Saturation assays were performed with \(10^{-2}\) M MDL 100,907 for 1.5 h at room temperature. Data represent the mean ± S.E.M. from three independent experiments.

<table>
<thead>
<tr>
<th>Transfection</th>
<th>(K_D) (nM)</th>
<th>(B_{\text{max}}) (fmol/mg)</th>
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<tr>
<td>(G_{11})</td>
<td>0.67 ± 0.05</td>
<td>14.30 ± 0.94</td>
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<tr>
<td>(G_{11}, S154D)</td>
<td>0.62 ± 0.08</td>
<td>12.84 ± 1.36</td>
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discussion
In the present study, we found that 24-h DOI treatment causes down-regulation of 5-HT\textsubscript{2A} receptors and uncoupling of \(G_{11}\) protein from 5-HT\textsubscript{2A} receptors in A1A1v cells. Our results also suggest that the increased phosphorylation of \(G_{11}\) protein induced by DOI treatment results in an uncoupling of G proteins from 5-HT\textsubscript{2A} receptors in A1A1v cells based on the use of a phosphorylation state mimic mutation, S154D, in \(G_{11}\) protein. However, the phosphorylation state mimic did not reduce agonist binding to 5-HT\textsubscript{2A} receptors. The additional effects of DOI treatment on receptor binding...
are consistent with our previous data suggesting that phosphorylation of \(G_{\alpha_{11}}\) protein contributes approximately half of the total desensitization response caused by DOI treatment (Shi et al., 2007).

Previous studies found that chronic administration of the 5-HT2A receptor agonist DOI resulted in reductions in the density of 5-HT2A receptors in the cortex (Anji et al., 2000; Hensler and Truett, 1998; McKenna et al., 1989). In our current study, we found that DOI treatment significantly lowered the \(B_{\text{max}}\) of \([3H]\)ketanserin-labeled total 5-HT2A receptor binding. These data suggest that down-regulation of 5-HT2A receptors was involved in DOI-induced desensitization in A1A1v cells. DOI treatment did not result in changes in the \(K_D\) value of either \([125I]\)(\pm\)DOI or \([3H]\)ketanserin binding, suggesting that the affinity of the 5-HT2A receptors was not altered by DOI treatment. Our current data show significant reductions in \([125I]\)(\pm\)DOI binding to 5-HT2A receptors following 24-h DOI treatment in A1A1v cells and that the 46.5% reduction in agonist binding is much greater than the 24.8% reduction in antagonist binding. These relative differences in agonist and antagonist binding are consistent with previous studies on rat frontal cortex in which agonist binding was reduced 86% and antagonist binding was reduced 51% (McKenna et al., 1989). Based on the ter-
normal rabbit IgG is also shown. A sample immunoprecipitated with saline for 21 days (S), or fluoxetine for 21 days (F). A sample immunoprecipitated with normal rabbit IgG is also shown.

Fig. 6. Effect of chronic fluoxetine treatment (10 mg/kg i.p. 21 days) on levels of phosphorylated $G_{\text{af11}}$ proteins. a, there was no significant effect of chronic fluoxetine treatment compared with saline-treated controls (Student’s t test). The data represent the mean ± S.E.M. of six rats per group. b, a representative blot of the samples treated with saline for 21 days (S), or fluoxetine for 21 days (F). A sample immunoprecipitated with normal rabbit IgG is also shown.

nary complex model, these results suggest that, in addition to overall reductions in 5-HT$_{2A}$ receptor density, DOI treatment causes a decreased coupling of 5-HT$_{2A}$ receptors with G proteins. The data from GTP$_{y}$S binding experiments demonstrate that GTP$_{y}$S-sensitive binding is decreased in cells treated with DOI in comparison with vehicle-treated cells, which further confirms the decreased coupling of 5-HT$_{2A}$ receptors with G proteins.

The decreased coupling of 5-HT$_{2A}$ receptors with G proteins may be due to the phosphorylation of 5-HT$_{2A}$ receptors, phosphorylation of G proteins, or a reduction in G protein levels. Results from previous studies (Damjanoska et al., 2004; Roth et al., 1995) suggest that the agonist-induced desensitization of 5-HT$_{2A}$ receptor signaling is not likely due to alterations in the levels of G proteins. Although phosphorylation of 5-HT$_{2A}$ receptors could result in the uncoupling of 5-HT$_{2A}$ receptors with G proteins and 5-HT$_{2A}$ receptors containing several consensus sites for effector kinases, protein kinase C and calcium-calmodulin dependent kinase II, an increase in the phosphorylation of 5-HT$_{2A}$ receptors by these enzymes during agonist-induced desensitization has yet to be shown (Gray et al., 2003).

To examine the hypothesis that DOI-induced phosphorylation of $G_{\text{af11}}$ results in the uncoupling of G protein with 5-HT$_{2A}$ receptors, cells were transfected with either the phosphorylation state mimic $G_{\text{af11S154D}}$ or wild-type $G_{\text{af11}}$. Forty-eight hours after transfection, the [$^{125}$I]$^\pm$-DOI-labeled high-affinity state 5-HT$_{2A}$ receptors were determined. If phosphorylation of $G_{\text{af11}}$ results in uncoupling of G protein from 5-HT$_{2A}$ receptors, according to the ternary complex model, we would expect to see a lower $B_{\text{max}}$ of [{$^{125}$I}]$^\pm$-DOI binding in cells transfected with $G_{\text{af11S154D}}$ compared with cells transfected with wild-type $G_{\text{af11}}$. We were unable to detect the difference in $B_{\text{max}}$ and $K_D$ value between cells transfected with wild-type $G_{\text{af11}}$ and $G_{\text{af11S154D}}$. However, GTP$_{y}$S-sensitive binding was decreased in cells transfected with phosphorylation state mimic $G_{\text{af11S154D}}$ ($p < 0.05$). One possible explanation of this result is that endogenous $G_{\text{af11S154D}}$ prevented detection of the difference, because the percentage of cells transiently transfected is approximately 40 to 60%. Another possibility is that, although 5-HT$_{2A}$ receptors exist in a high-affinity-$G_{\alpha}$ protein coupled state (Battaglia et al., 1984), some receptors can exist in a high-affinity state that is not coupled to $G_{\alpha}$ as suggested by a revised ternary model for 5-HT$_{2A}$ receptors (Egan et al., 2000; Roth et al., 1997). Based on the revised ternary model, GTP$_{y}$S-sensitive binding would be the better index of receptor-G protein coupling. In cells transfected with either wild-type or mutant $G_{\text{af11}}$ proteins, we found some agonist binding remaining after adding GTP$_{y}$S, suggesting that there is a population of 5-HT$_{2A}$ receptor proteins in the membrane that is able to bind agonist but is not G protein-coupled, consistent with the revised ternary model for 5-HT$_{2A}$ receptors (Egan et al., 2000; Roth et al., 1997).

Phosphorylation of $G_{\text{af11}}$ protein could hinder the coupling to 5-HT$_{2A}$ receptors by either directly interrupting the interaction of $G_{\text{af11}}$ with 5-HT$_{2A}$ receptors or by directly interrupting the interaction of $G_{\beta}\gamma$ with $G_{\text{af11}}$ protein and thereby preventing the formation of the heterotrimeric G protein complex, which binds to 5-HT$_{2A}$ receptors. Phosphorylation of tyrosine residues in $G_{\text{af11}}$ proteins disrupts the interaction of $G_{\text{af11}}$ proteins with M$_3$ muscarinic receptors (Umemori et al., 1997). Protein kinase C-mediated phosphorylation of $G_{\alpha_1}$ prevents association with $G_{\beta\gamma}$, and conversely association of $G_{\alpha_1}$ with $G_{\beta\gamma}$ prevents phosphorylation in vitro (Fields and Casey, 1995). However, $G_{\text{oxz}}$ is primarily phosphorylated at Ser27 and is also phosphorylated at Ser16. Likewise, protein kinase C-mediated phosphorylation of $G_{\text{ozx}}$ in cells and in vitro is prevented by its interaction with $G_{\beta\gamma}$ (Kozasa and Gilman, 1986). However, $G_{\text{ozx12}}$ is also phosphorylated in the amino terminus of the protein, possibly within the first 50 amino acids based on digestion experiments. Previous studies have also shown that the amino terminus of $G_{\text{oxz12}}$ is important for binding to $G_{\beta\gamma}$, especially isoleucine 25 (Evanko et al., 2005). These results make decreased binding of $G_{\beta\gamma}$ to Ser154-phosphorylated $G_{\text{af11S154D}}$ a less likely possibility. Previous studies have also found that phosphorylation of $G_{\alpha_1}$ proteins can reduce binding to other proteins. For example, phosphorylation of $G_{\text{ozx12}}$ inhibits interaction with the effector enzyme (Strassheim and Malbon, 1994), and phosphorylation of $G_{\text{oxz}}$ inhibits interaction with regulator of G protein signaling (RGS) proteins (Glick et al., 1998).

In vivo, sustained treatment with DOI decreases the maximal response ($B_{\text{max}}$) of 5-HT-stimulated PLC activity and increases the phosphorylation of $G_{\text{af11}}$ proteins. Maximal 5-HT-stimulated PLC activity was reduced in frontal cortex by 32% after 4 days and 40% after 7 days of DOI treatment. In the frontal cortex, phosphorylated $G_{\text{af11}}$ proteins increase gradually and are 31% above control levels after 4 days and...
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References


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