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Characterization of the Functional Heterologous Desensitization of Hypothalamic 5-HT_{1A} Receptors after 5-HT_{2A} Receptor Activation

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Desensitization of 5-HT_{1A} receptors could be involved in the long-term therapeutic effect of anxiolytic and antidepressant drugs. Pretreatment of rats with the 5-$HT_{2A/2C}$ agonist DOI induces an attenuation of hypothalamic 5-HT_{1A} receptor–G_z-protein signaling, measured as the ACTH and oxytocin responses to an injection of the 5-HT_{1A} agonist 8-OH-DPAT. We characterized this functional heterologous desensitization of 5-HT_{1A} receptors in rats and examined some of the mechanisms that are involved. A time course experiment revealed that DOI produces a delayed and reversible reduction of the ACTH and oxytocin responses to an 8-OH-DPAT challenge. The maximal desensitization occurred at 2 hr, and it disappeared 24 hr after DOI injection. The desensitization was dose-dependent, and it shifted the oxytocin and ACTH dose–response curves of 8-OH-DPAT to the right (increased ED_{50}) with no change in their maximal responses (E_{max}). The 5-HT_{2A} receptor antagonist MDL 100,907 prevented the DOI-induced desensitization, indicating that 5-HT_{2A} receptors mediate the effect of DOI. Analysis of the components of the 5-HT_{1A} receptor–G_z-protein signaling system showed that DOI did not alter the level of membrane-associated G_z-proteins in the hypothalamus. Additionally, DOI did not alter the binding of [^{3}H]8-OH-DPAT or the inhibition by GTPγS of [^{3}H]8-OH-DPAT binding in the hypothalamus. In conclusion, the activation of 5-HT_{2A} receptors induces a transient functional desensitization of 5-HT_{1A} receptor signaling in the hypothalamus, which may occur distal to the 5-HT_{1A} receptor–G_z-protein interface.

Key words: neuroendocrine; serotonin; oxytocin; ACTH; G_z-protein; [^{3}H]8-OH-DPAT binding; GTPγS

Among the seven families of serotonin receptors, 5-HT_{1A} and 5-HT_{2A} receptors have an important role in mood disorders (Stockmeier et al., 1997; Staley et al., 1998; Olivier et al., 1999; Sargent et al., 2000). Selective serotonin reuptake inhibitors (SSRIs) produce a desensitization of 5-HT_{1A} receptors (Li et al., 1996; Berlin et al., 1998; Raap et al., 1999; Bosker et al., 2001) and alter the functioning of 5-HT_{2A} receptors (Tilakaratne et al., 1995; Bonson et al., 1996; Raap and Van de Kar, 1999). Desensitization of 5-HT_{1A} receptors could contribute to the therapeutic effects of SSRIs. Because chronic treatment with SSRIs results in long-term elevation in the levels of 5-HT in the synapse, SSRIs could produce a heterologous desensitization of 5-HT_{1A} receptors by the activation of other 5-HT receptors.

5-HT_{1A} receptors and 5-HT_2 receptors interact via their signaling proteins (Katada et al., 1985; Berg et al., 1998; Tournois et al., 1998; Evans et al., 2001). In Chinese hamster ovary (CHO) cells stably expressing the human 5-HT_{1A} receptors and 5-HT_{2C} receptors, the activation of 5-HT_{2C} receptors induces a heterologous desensitization of 5-HT_{1A} receptors via protein kinase C and a cyclooxygenase-dependent metabolite of arachidonic acid (Evans et al., 2001). 5-HT_{2A} receptors are coupled also via $G_{q/11}$ proteins to phospholipase C and phospholipase A_2 signaling pathways (Garcia and Kim, 1997; Grotewiel and Sanders-Bush, 1999). Thus 5-HT_{2A} receptors could cross-talk to 5-HT_{1A} receptors to produce a heterologous desensitization of 5-HT_{1A} receptors.

Functional interactions between 5-HT_{1A} receptors and 5-$HT_{2A/2C}$ receptors have been demonstrated in behavioral studies. These studies either examined the effect of 5-HT_{1A} receptor activation on 5-$HT_{2A/2C}$ receptor-mediated behaviors (Darmani et al., 1990; Krebs-Thomson and Geyer, 1998) or determined the impact of 5-$HT_{2A/2C}$ receptor desensitization on the behavioral and temperature responses to 5-HT_{1A} receptor activation (Hensler and Truett, 1998). To our knowledge, only one group has examined the impact of 5-$HT_{2A/2C}$ receptor activation on the reproductive behavioral response to 8-OH-DPAT (Maswood and Uphouse, 1997).

5-HT_{1A} receptors labeled with [^{3}H]8-OH-DPAT and 5-$HT_{2A/2C}$ receptors labeled with [I^{125}]DOI are found in the hypothalamic paraventricular nucleus (PVN) (Appel et al., 1990; Li et al., 1997), where oxytocin and corticotropin-releasing factor (CRF) cells are located (Sawchenko and Swanson, 1985). Evidence indicates that 8-OH-DPAT-induced release of oxytocin and adrenocorticotropic hormone (ACTH) is mediated by 5-HT_{1A} receptor–G_z-protein signaling in the hypothalamic paraventricular nucleus (Serres et al., 2000). Activation of hypothalamic 5-HT_{2A} receptors also mediates oxytocin and ACTH release (Bagdy 1996; Van de Kar et al., 2001). Thus the 8-OH-DPAT-induced increase in the plasma levels of ACTH and oxytocin is a useful index of the sensitivity of hypothalamic

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5-HT$_{1A}$ receptors and can be used to examine the interaction between 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors in the hypothalamus.

The present study is the first *in vivo* characterization of a functional heterologous desensitization of 5-HT$_{1A}$ receptors after 5-HT$_{2A}$ receptor activation with DOI. In addition, the hypothalamic levels of membrane-associated G_z-protein and the coupling of hypothalamic 5-HT$_{1A}$ receptors to G-proteins were examined as possible underlying mechanisms for this heterologous desensitization.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (225–275 gm) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The rats were housed two per cage in a temperature-, humidity-, and light-controlled room (12 hr light/dark cycle, lights on from 7:00 A.M. to 7:00 P.M.). Food and water were available *ad libitum*. All procedures were conducted in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* as approved by Loyola University Institutional Animal Care and Use Committee.

Drugs

±8-Hydroxy-2-(di-*n*-propylamino) tetralin hydrobromide ([^{3}H]8-OH-DPAT) and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI) were purchased from Research Biochemicals (Natick, MA). (±)-α-(2,3-Dimethoxyphenyl)-1-(2-fluorophenylethyl-4-piperidinemethanol (MDL 100,907) was donated generously by Hoechst Marion Roussel Research Institute (Cincinnati, OH). 8-OH-DPAT was dissolved in 0.9% saline at four concentrations (0.03, 0.05, 0.1, and 0.5 mg/ml). DOI was dissolved in 0.9% saline at three concentrations (0.5, 2.5, and 5 mg/ml). MDL 100,907 was dissolved in a minimal volume of 0.01N HCl and diluted with saline to the final concentration of 0.01 mg/ml. All solutions were made fresh before injections and injected at a volume of 1 ml/kg.

Experimental protocols

After their arrival, the rats were housed two per cage for at least 1 week, followed by 4 d of handling. On the day of the experiments the rats were assigned randomly to different experimental groups ($n = 8$ per group) to receive drug treatments. Cage mates were assigned to the same treatment group. After receiving different drug treatments, the rats were decapitated. The trunk blood was collected in centrifuge tubes containing a 0.5 ml solution of 0.3 M EDTA, pH 7.4. After centrifugation the plasma was stored at −70°C for radioimmunoassays of plasma hormone concentrations. The hypothalamus was dissected, immediately frozen in liquid nitrogen, and later stored at −70°C for immunoblot analysis and receptor binding assays.

Time course of the effect of DOI on hormone responses to an 8-OH-DPAT challenge. The 5-HT$_{2A/2C}$ receptor agonist DOI (2.5 mg/kg, i.p.) or saline was administered to rats. At different time points after DOI injection (1, 2, 4, 24 hr) the rats were challenged with the 5-HT$_{1A}$ agonist 8-OH-DPAT (0.05 mg/kg, s.c.) or saline. Then 15 min after the 8-OH-DPAT injection the rats were decapitated.

Effect of pretreatment with increasing doses of DOI on hormone responses to an 8-OH-DPAT challenge. Rats were injected with increasing doses of DOI (0.5, 2.5 and 5 mg/kg, i.p.) or saline. At 2 hr later the rats were challenged with 8-OH-DPAT (0.05 mg/kg, s.c.) or saline. Then 15 min after the 8-OH-DPAT challenge the rats were decapitated.

Dose–response to an 8-OH-DPAT challenge in rats pretreated with DOI. Rats were injected with DOI (2.5 mg/kg, i.p.) or saline. At 2 hr later the rats were challenged with saline or increasing doses of 8-OH-DPAT (0.03, 0.05, 0.1, 0.5 mg/kg, s.c.). Then 15 min after the 8-OH-DPAT challenge the rats were decapitated.

Pretreatment with the 5-HT$_{2A}$ antagonist MDL 100,907. Rats first received an injection of vehicle or the 5-HT$_{2A}$ antagonist MDL 100,907 (0.01 mg/kg, s.c.). At 15 min after this injection the rats received an injection of DOI (2.5 mg/kg, i.p.) or saline. Then 2 hr after the DOI injection the rats were challenged with 8-OH-DPAT (0.05 mg/kg, s.c.) or saline. The rats were decapitated 15 min after the 8-OH-DPAT challenge.

Radioimmunoassay

Plasma oxytocin and ACTH were determined by radioimmunoassays as described previously in detail (Li et al., 1993, 1997).

Immunoblot analysis of G_z-proteins

Tissue preparation. All procedures were conducted at 4°C. Briefly, the hypothalamic tissues were homogenized in 0.4 ml of 50 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 10% sucrose, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and additional protease inhibitors purchased as a cocktail [containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, bestatin, leupeptin, and aprotinin] from Sigma (1.5 μl/30 mg tissue; St. Louis, MO). After centrifugation at 20,000 × *g* for 60 min the pellets were collected and resuspended by sonication in a 20 mM Tris buffer, pH 8 [containing (in mM) 1 EDTA, 100 NaCl, 1 dithiothreitol, and 1% sodium cholate] plus the protease inhibitory cocktail (1.5 μl cocktail/30 mg tissue) in a ratio of 3 μl buffer/mg tissue. The resuspended pellets were incubated while shaking for 1 hr and then centrifuged at 100,000 × *g* for 60 min. The supernatant was collected for the immunoblot analysis of membrane-bound G_z-protein levels. The protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Quantification of G_z-protein. Immunoblot analysis of membrane-associated G_z-proteins has been described previously in detail (Raap et al., 1999; Serres et al., 2000). Briefly, the solubilized proteins (2 μg/lane) were resolved by SDS-PAGE and then transferred electrophoretically to nitrocellulose membranes. After incubation with a blocking buffer (PBS containing 0.2% casein and 0.1% Tween 20), the nitrocellulose membranes were probed overnight at 4°C with polyclonal antisera for G_z (I-20, 1:6000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Then the membranes were incubated with a secondary antibody (goat anti-rabbit serum, 1:25,000 dilution for 1 hr; Cappell, Organon Teknika, Durham, NC), followed by an incubation with rabbit peroxidase anti-peroxidase (1:5000 dilution for 1 hr; Cappell, Organon Teknika). Finally, the membranes were incubated with the ECL chemiluminescence substrate solution (Amersham, Arlington Heights, IL) and then exposed to Kodak x-ray film. Films were analyzed densitometrically with the Scion Image program (Frederick, MD). The data for each sample were the means of three replications and were calculated as integrated optical densities (IOD)/μg protein. The percentile changes of the mean IOD/μg protein values of each sample were calculated with respect to the average of IOD/μg protein values of saline-treated samples.

[^{3}H]8-OH-DPAT binding

All procedures were performed on ice. The hypothalamic tissues were homogenized quickly in 2 ml Tris buffer (50 mM), pH 7.7, using a Polytron, and were centrifuged at 35,000 × *g* for 10 min. The pellets were resuspended with 2 ml of 50 mM Tris buffer, and the procedure was repeated four times. Then the samples were diluted to a final concentration of 20 mg of tissue per milliliter for the receptor binding assay. [^{3}H]8-OH-DPAT binding (specific activity, 124.9 mCi/ml, 0.8 nM) was performed in 1 ml of 50 mM Tris buffer, pH 7.7, containing 0.5 mM EDTA, 10 mM $MgSO_4$, 0.8 nM [^{3}H]8-OH-DPAT, 2 mg hypothalamic tissue, and different concentrations of GTPγS (from 10^{-9} to 10^{-5} M). The specificity of [^{3}H]8-OH-DPAT binding to 5-HT$_{1A}$ receptors was defined in the presence of 1 μM WAY100635. The ability of GTPγS at both IC_{50} (0.02 μM) and E_{max} (1 μM) concentrations to inhibit [^{3}H]8-OH-DPAT labeling of 5-HT$_{1A}$ receptors was examined in hypothalamic homogenates obtained from saline- and DOI-treated (2.5 mg/kg, i.p.) rats. The protein concentration was measured with a BCA protein assay kit (Pierce). 5-HT$_{1A}$ receptor binding was expressed as femtomoles per milligram of protein.

Statistical analyses

The data are presented as group means ($n = 8$) and the SEM. Except for immunoblot data, all other data were analyzed by two-way or three-way ANOVA. Group means were compared by a Newman–Keuls multiple range test (Steel and Torrie, 1960). The immunoblot data were analyzed by a Student's *t* test. A computer program (GBSTAT, Silver Spring, MD) was used for all of the statistical analyses.

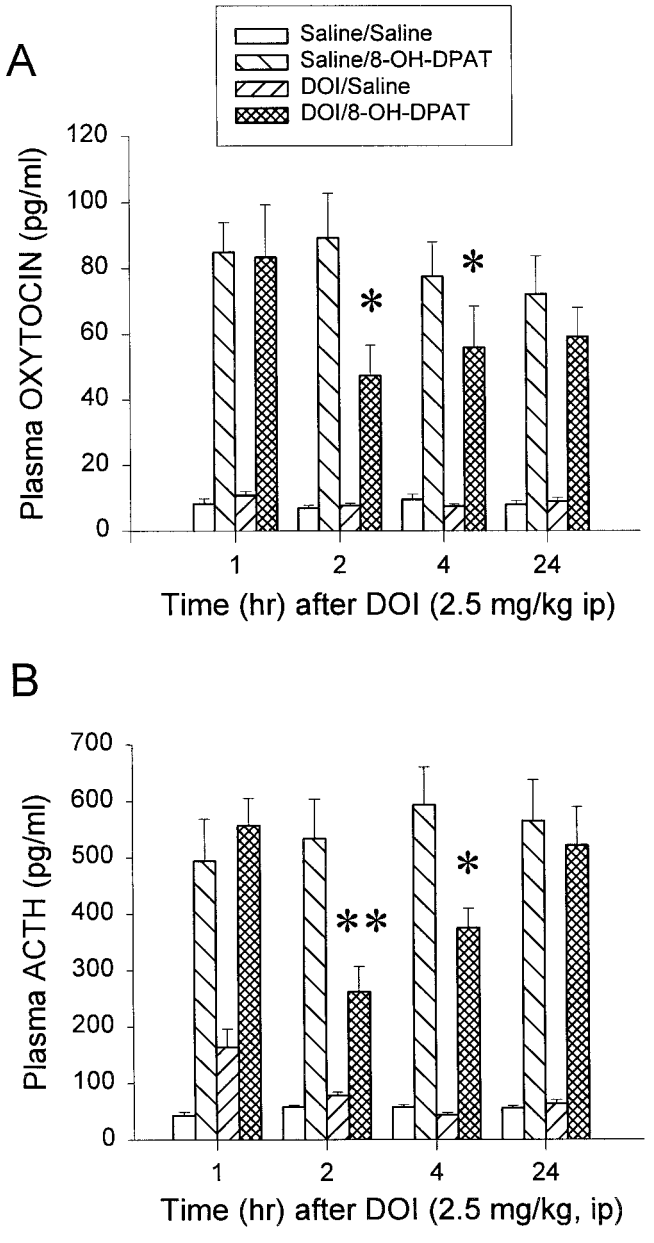

Figure 1. Time course of DOI-induced reduction of the oxytocin (*A*) and the ACTH (*B*) responses to an 8-OH-DPAT (0.05 mg/kg, s.c.) challenge. The data represent the means ± SEM of six to eight rats per group. *Significant difference from saline/8-OH-DPAT group ($p < 0.05$); **significant difference from saline/8-OH-DPAT group ($p < 0.01$); three-way ANOVA and Newman–Keuls multiple range test.

RESULTS

Time course of the effect of DOI on hormone responses to a challenge with a 5-HT$_{1A}$ agonist

The 5-HT$_{2A/2C}$ receptor agonist DOI (2.5 mg/kg, i.p.) was injected at different time points (1, 2, 4, 24 hr) before 8-OH-DPAT challenge. DOI alone did not alter plasma oxytocin and ACTH levels significantly at these time points. 8-OH-DPAT significantly increased the plasma levels of oxytocin and ACTH by 731 and 930%, respectively. The time course effects of DOI on oxytocin (Fig. 1*A*) and ACTH (Fig. 1*B*) showed a delayed onset and reversible inhibition, by DOI, of the hormone responses to the subsequent challenge with 8-OH-DPAT. For oxytocin the three-way ANOVA indicated no significant main effects of time ($F_{(3,107)} = 0.04$; $p > 0.1$) but a significant main effect of DOI ($F_{(1,107)} = 9.96$; $p < 0.01$) and 8-OH-DPAT ($F_{(1,107)} = 270.51$; $p < 0.01$). The interaction between time and DOI was not significant. However, there was a significant interaction between DOI and 8-OH-DPAT ($F_{(1,107)} = 10.55$; $p < 0.01$). For ACTH the three-way ANOVA indicated that there was a significant main effect of 8-OH-DPAT ($F_{(1,106)} = 319.56$; $p < 0.01$). The main effect of time ($F_{(3,106)} = 2.45$; $p > 0.05$) and the main effect of DOI ($F_{(1,106)} = 3.23$; $p > 0.05$) were not significant. However, both the interactions between time and DOI ($F_{(3,106)} = 4.75$; $p < 0.05$) and between DOI and 8-OH-DPAT ($F_{(1,106)} = 10.48$; $p < 0.05$) were significant. The Newman–Keuls test indicated that DOI significantly decreased both plasma oxytocin and ACTH responses to the 8-OH-DPAT challenge at 2 and 4 hr, with a maximal effect at 2 hr post-DOI administration (47% reduction for oxytocin; 51% reduction for ACTH) (Fig. 1). At 1 hr after treatment with DOI there was no inhibition of the effect of 8-OH-DPAT on either ACTH or oxytocin levels (Fig. 1). These observations indicate that activation of 5-HT$_{2A/2C}$ receptors produces a transient and delayed onset attenuation of 5-HT$_{1A}$ receptor-mediated secretion of oxytocin and ACTH.

Effect of pretreatment with increasing doses of DOI on hormone responses to 8-OH-DPAT

DOI was administered at three doses: 0.5, 2.5, and 5 mg/kg intraperitoneally, respectively. Basal plasma levels of oxytocin and ACTH in saline-challenged rats were not altered significantly by DOI at these doses. However, DOI inhibited the oxytocin (Fig. 2*A*) response to 8-OH-DPAT challenge in a dose-dependent manner. For plasma oxytocin the two-way ANOVA indicated a significant main effect of both DOI ($F_{(3,55)} = 2.92$; $p < 0.05$) and 8-OH-DPAT ($F_{(1,55)} = 71.08$; $p < 0.01$). There was a significant interaction between DOI and 8-OH-DPAT ($F_{(3,55)} = 3.08$; $p < 0.05$). The Newman–Keuls test indicated that DOI significantly reduced the effect of 8-OH-DPAT on plasma oxytocin levels at the doses of 2.5 mg/kg (by 40%; $p < 0.05$) and 5 mg/kg (by 58%; $p < 0.01$). For plasma ACTH in this experiment the two way ANOVA indicated a significant main effect of 8-OH-DPAT ($F_{(1,54)} = 119.82$; $p < 0.01$), but no significant main effect of DOI ($F_{(3,54)} = 0.25$; $p > 0.1$) and no significant interaction between DOI and 8-OH-DPAT ($F_{(3,54)} = 1.47$; $p > 0.1$). However, baseline levels of ACTH were elevated from 43.1 ± 3.4 to 99.6 ± 12.8. Subtracting the baseline levels from 8-OH-DPAT-stimulated ACTH release would indicate that the additive effects of DOI on basal ACTH levels could have masked its desensitizing effects (saline/DPAT group, 382.2; DOI 0.5/DPAT group, 326.6; DOI 2.5/DPAT group, 271.2; DOI 5/DPAT group, 228.4 pg/ml). Hence the highest dose of DOI produced a >40% inhibition of the ACTH response to 8-OH-DPAT.

Dose–response effect of 8-OH-DPAT in rats pretreated with DOI

Increasing doses of 8-OH-DPAT (0.03, 0.05, 0.1, 0.5 mg/kg, s.c.) or saline were administered 2 hr after DOI (2.5 mg/kg, i.p.) or saline treatments. In saline-pretreated rats, 8-OH-DPAT significantly increased plasma oxytocin and ACTH levels in a dose-dependent manner. At the doses of 0.05 and 0.1 mg/kg, 8-OH-DPAT increased plasma oxytocin to 53 and 80% of maximal response, respectively. However, ACTH is a more amplified hormone. At the dose of 0.05 mg/kg, 8-OH-DPAT produced a maximal increase in plasma ACTH levels. DOI treatment shifted

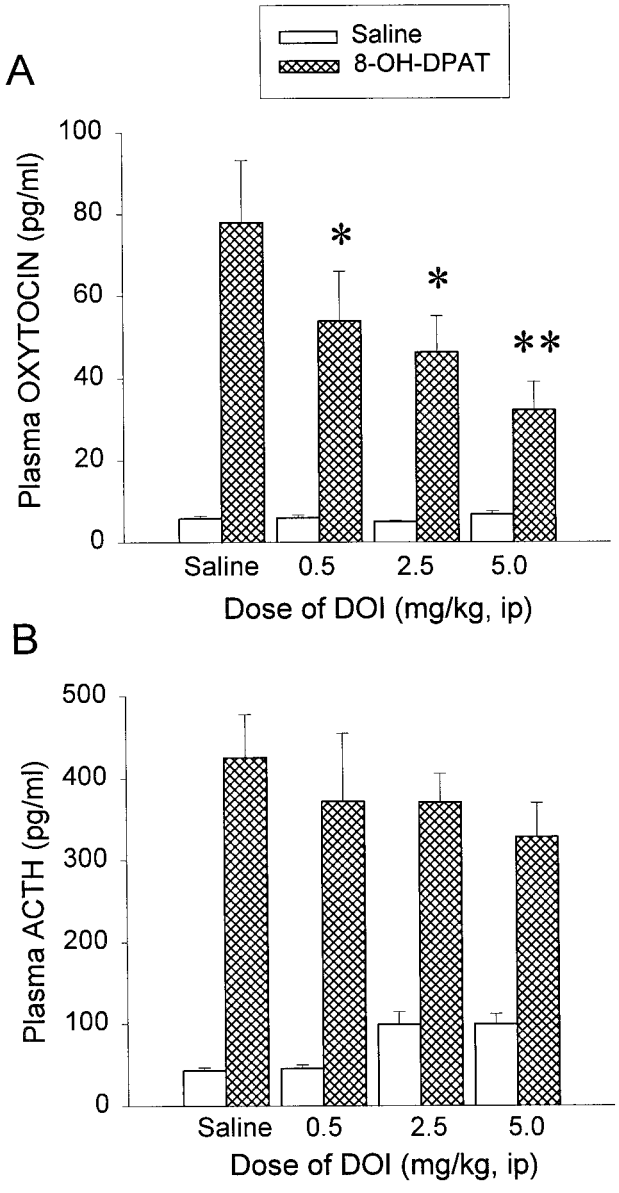

Figure 2. Examination of the doses of DOI that reduce the oxytocin (*A*) and the ACTH (*B*) responses to an 8-OH-DPAT (0.05 mg/kg, s.c.) challenge. The data represent the means ± SEM of seven to eight rats per group. *Significant difference from saline/8-OH-DPAT group ($p < 0.05$); **significant difference from saline/8-OH-DPAT group ($p < 0.01$); two-way ANOVA and Newman–Keuls multiple range test.

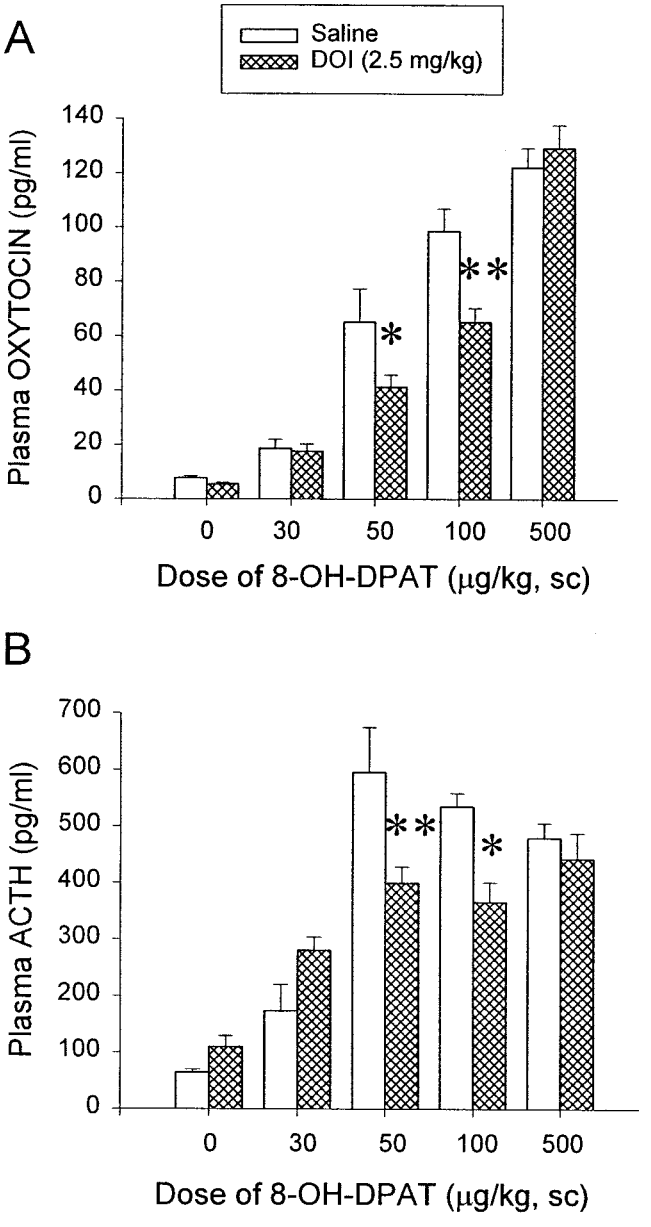

Figure 3. DOI shifts the oxytocin (*A*) and ACTH (*B*) dose–response curve of 8-OH-DPAT effects to the right, with no change in E_{max}. The data represent the means ± SEM of six to eight rats per group. *Significant difference from saline/8-OH-DPAT group ($p < 0.05$); **significant difference from saline/8-OH-DPAT group ($p < 0.01$); two-way ANOVA and Newman–Keuls multiple range test.

both oxytocin (Fig. 3*A*) and ACTH (Fig. 3*B*) dose–response curves to the right, with no change in E_{max}. For plasma oxytocin the two-way ANOVA showed a significant main effect of DOI ($F_{(1,67)} = 7.26$; $p < 0.01$) and a significant main effect of 8-OH-DPAT ($F_{(4,67)} = 120.00$; $p < 0.01$). The interaction between DOI and 8-OH-DPAT was also significant ($F_{(4,67)} = 3.72$; $p < 0.01$). The Newman–Keuls test indicated that, in saline-pretreated rats, 8-OH-DPAT at the dose of 0.03 mg/kg produced no significant effect. At the doses of 0.05 and 0.1 mg/kg, 8-OH-DPAT significantly elevated plasma oxytocin levels. Basal plasma oxytocin level in saline-challenged rats was not altered by DOI. However, DOI significantly lowered the oxytocin responses to 8-OH-DPAT at the challenge doses of 0.05 mg/kg ($p < 0.05$) and 0.1 mg/kg ($p < 0.01$), but not at the highest dose of 0.5 mg/kg (Fig. 3*A*). For plasma ACTH the two-way ANOVA indicated a significant main effect of DOI ($F_{(1,60)} = 4.67$; $p < 0.05$) and a significant main effect of 8-OH-DPAT ($F_{(4,60)} = 47.7$; $p < 0.01$). There was a significant interaction between DOI and 8-OH-DPAT ($F_{(4,60)} = 6.45$; $p < 0.01$). The Newman–Keuls test indicated that 0.03 mg/kg 8-OH-DPAT produced no significant effect on plasma ACTH. However, a dose of 0.05 mg/kg 8-OH-DPAT already increased plasma ACTH to its maximal level. Basal plasma ACTH level in saline-challenged rats was not altered by DOI. However, DOI significantly lowered the ACTH response to 8-OH-DPAT at the challenge doses of 0.05 mg/kg ($p < 0.01$) and 0.1 mg/kg ($p < 0.05$), respectively, but not at the dose of 0.5 mg/kg (Fig. 3*B*).

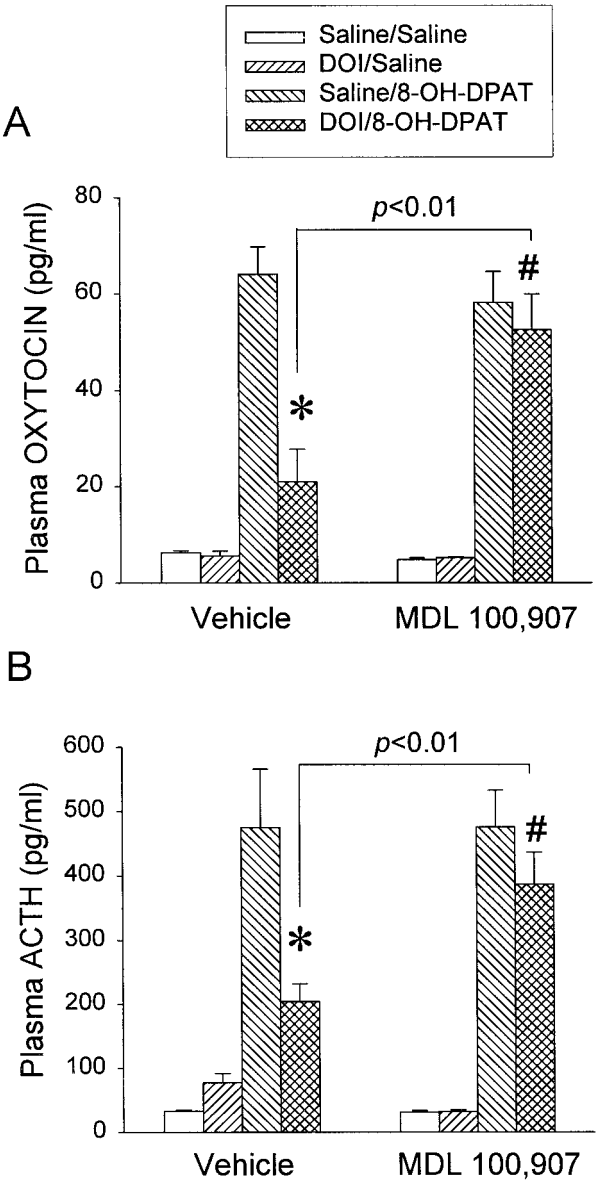

Figure 4. The 5-HT$_{2A}$ receptor antagonist MDL 100,907 reverses the inhibitory effect of DOI on the oxytocin (*A*) and the ACTH (*B*) responses to an 8-OH-DPAT (0.05 mg/kg, s.c.) challenge. The data represent the means ± SEM of 7–10 rats per group. *Significant difference from saline/8-OH-DPAT group ($p < 0.01$); #significant difference from DOI/8-OH-DPAT group ($p < 0.01$); three-way ANOVA and Newman–Keuls multiple range test.

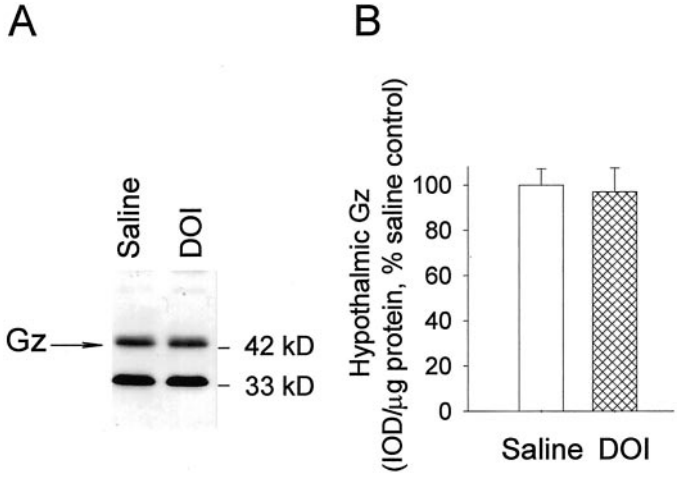

Figure 5. DOI does not change the level of membrane-associated G$_z$-protein in the hypothalamus. *A*, Immunoblot of G$_z$-proteins in the particulate fraction of the hypothalamic from DOI- and saline-treated rats. *B*, Results of the densitometric analysis of the membrane-bound G$_z$-proteins in the hypothalamus. The data represent the means ± SEM of eight hypothalamic samples per group. The data for each sample are the average of three replicates and were analyzed with a Student's *t* test.

Pretreatment with the 5-HT$_{2A}$ antagonist MDL 100,907 prevents DOI-induced reduction of hormone responses to 8-OH-DPAT

The basal plasma levels of oxytocin and ACTH were not altered by MDL 100,907 or by DOI. The 8-OH-DPAT challenge significantly elevated both plasma levels of oxytocin (Fig. 4*A*) and ACTH (Fig. 4*B*). The three-way ANOVA indicated that there was no significant main effect of MDL 100,907 for oxytocin ($F_{(1,55)} = 2.74$; $p > 0.1$) and ACTH ($F_{(1,55)} = 1.08$; $p > 0.1$). For oxytocin there were significant interactions between MDL 100,907 and DOI ($F_{(1,55)} = 7.17$; $p < 0.01$) as well as among MDL 100,907, DOI, and 8-OH-DPAT ($F_{(1,55)} = 6.41$; $p < 0.05$). For ACTH the main effect of 8-OH-DPAT was significant ($F_{(1,55)} = 109.11$; $p < 0.01$), but neither the interaction between MDL 100,907 and DOI nor the interaction among MDL 100,907, DOI, and 8-OH-DPAT was significant. However, the Newman–Keuls test indicated that MDL 100,907 blocked the ability of DOI to inhibit the effect of 8-OH-DPAT on plasma levels of oxytocin (Fig. 4*A*) and ACTH (Fig. 4*B*). Thus the effect of DOI on both hormone responses to 8-OH-DPAT challenge is mediated predominantly by the activation of 5-HT$_{2A}$ receptors.

DOI does not change the level of membrane-associated G$_z$-proteins in the hypothalamus

The concentration of membrane-associated G$_z$-proteins in the hypothalamus of DOI- and saline-treated rats was measured by immunoblot analysis. The specificity of the antibody for G$_z$-protein has been verified in a previous study (Raap et al., 1999). An example of an immunoblot of membrane-bound G$_z$-proteins is shown in Figure 5*A*. The result of the densitometric analysis is presented in Figure 5*B*. A Student's *t* test indicates that hypothalamic levels of membrane-associated G$_z$-proteins were not reduced significantly by 2.5 mg/kg DOI intraperitoneally, a dose that significantly reduces hormone responses to an 8-OH-DPAT challenge.

DOI does not change the GTPγS-induced inhibition of [^{3}H]8-OH-DPAT binding in the hypothalamus

5-HT$_{1A}$ receptors in the hypothalamus were labeled with [^{3}H]8-OH-DPAT (0.8 nM). The specific binding of [^{3}H]8-OH-DPAT was essentially the same when 1 μM 5-HT or 1 μM WAY100635 was used to define nonspecific binding. Increasing concentrations of GTPγS (from 10^{-9} to 10^{-5} M) gradually inhibited [^{3}H]8-OH-DPAT binding with an $EC_{50} \approx 0.02$ μM and an E_{max} concentration ≈1 μM (Fig. 6*A*).

We used 0.02 μM (IC_{50}) and 1 μM (E_{max}) concentrations of GTPγS to examine the degree of inhibition of [^{3}H]8-OH-DPAT (0.8 nM) binding in the hypothalamic homogenates from saline- and DOI-treated rats. The two-way ANOVA indicated that there was a significant main effect of GTPγS ($F_{(42,2)} = 374.54$; $p < 0.01$), but no significant main effect of DOI ($F_{(42,1)} = 2.45$; $p > 0.1$). The interaction between GTPγS and DOI was not signifi-

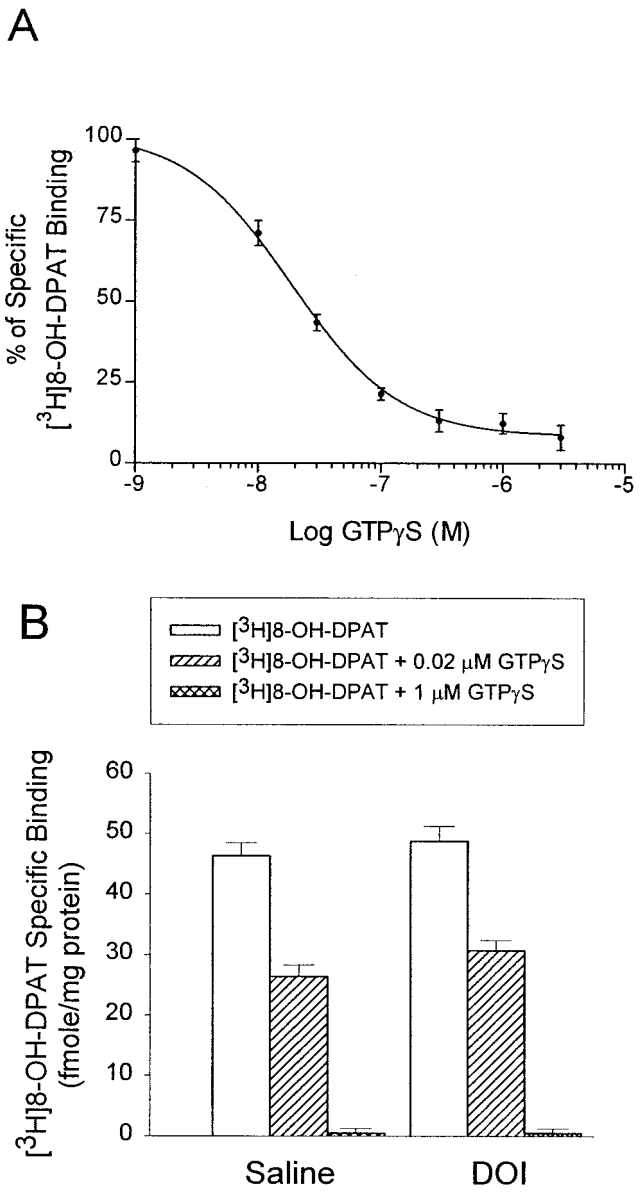

Figure 6. DOI does not change the coupling of 5-HT$_{1A}$ receptors to G-proteins in the hypothalamus. *A*, Inhibition of [^{3}H]8-OH-DPAT (0.8 nM) binding in hypothalamic homogenates with increasing concentration of GTPγS (from 10^{-9} to10^{-5} M). Each *data point* represents the mean of three assays. Specific binding is defined by WAY100635 (1 μM). *B*, DOI does not alter the GTPγS-induced inhibition of [^{3}H]8-OH-DPAT binding at the GTPγS concentrations of 0.02 μM (EC_{50}) and 1 μM (E_{max}). The data represent the means ± SEM of [^{3}H]8-OH-DPAT binding from eight hypothalamic samples per group; two-way ANOVA and Newman–Keuls multiple range test.

cant ($F_{(42,2)} = 0.74$; $p > 0.1$). The Newman–Keuls test indicated that DOI treatment did not alter the ability of both concentrations of GTPγS (0.02 or 1 μM) to inhibit the binding of [^{3}H]8-OH-DPAT in hypothalamic homogenates (Fig. 6*B*). These data suggest that DOI treatment does not reduce the coupling of 5-HT$_{1A}$ receptors to G-proteins in the hypothalamus.

DISCUSSION

The present study provides the first *in vivo* demonstration of a functional heterologous desensitization of hypothalamic 5-HT$_{1A}$ receptors after activation of 5-HT$_{2A}$ receptors. The main findings are that (1) activation of 5-HT$_{2A}$ receptors with DOI produces a delayed and transient reduction of 5-HT$_{1A}$ receptor-mediated secretion of oxytocin and ACTH, with an increase in ED_{50} and no change in E_{max}, and that (2) DOI treatment does not change the level of membrane-associated G_z-protein or the coupling of 5-HT$_{1A}$ receptors to G-proteins in the hypothalamus.

8-OH-DPAT, the prototypical 5-HT$_{1A}$ receptor agonist, has a high affinity for 5-HT$_{1A}$ receptors and from 10- to 100-fold lower affinity for other 5-HT receptor subtypes (Hoyer et al., 1994). The effect of 8-OH-DPAT on the secretion of ACTH and oxytocin is inhibited by the 5-HT$_{1A}$ antagonists WAY100635, NAN-190, and pindolol (Bagdy and Kalogeras, 1993; Critchley et al., 1994; Meller and Bohmaker, 1994; Vicentic et al., 1998). Thus the changes in plasma levels of ACTH and oxytocin after an 8-OH-DPAT challenge reflect the functional state of the 5-HT$_{1A}$ receptor system.

DOI is a 5-HT$_{2A/2C}$ agonist with a similar affinity for 5-HT$_{2A}$ and 5-HT $_{2C}$ receptors (Hoyer, 1988). Therefore, we used MDL 100,907 to determine whether 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors mediate the DOI-induced desensitization of 5-HT$_{1A}$ receptors. MDL 100,907 is a selective antagonist with >100-fold higher affinity for 5-HT$_{2A}$ than for 5-HT$_{2C}$ receptors (Johnson et al., 1996; Kehne et al., 1996). The dose of MDL 100,907 (0.01 mg/kg) used in the present study was selected to avoid occupancy of 5-HT$_{2C}$ receptors (Dekeyne et al., 1999; Smith et al., 1999). Thus the ability of MDL 100,907 to block the effect of DOI indicates that 5-HT$_{2A}$ receptors mediate the DOI-induced desensitization of hypothalamic 5-HT$_{1A}$ receptors.

Although evidence exists for receptor reserve in 5-HT$_{1A}$ receptor-induced release of ACTH (Meller and Bohmaker, 1994), oxytocin is a more direct indicator of the functioning of the hypothalamic 5-HT$_{1A}$ receptors after 8-OH-DPAT challenge (Serres et al., 2000). A dose of 0.05 mg/kg 8-OH-DPAT produces a maximal release of ACTH but only 50% of the maximal response of oxytocin (Fig. 3). This difference in amplification may explain our observation that DOI attenuates the oxytocin response to 8-OH-DPAT in a dose-dependent manner (Fig. 2*A*), whereas the inhibition of the ACTH response to 8-OH-DPAT is less dramatic (Fig. 2*B*).

DOI increases the secretion of oxytocin and ACTH by activating 5-HT$_{2A}$ receptors in the hypothalamic paraventricular nucleus (Van de Kar et al., 2001). It could be argued that a reduction of hormone responses to a subsequent 8-OH-DPAT challenge is attributable to a hormone-depleting effect of DOI. However, the time course experiment indicates that, 1 hr after treatment with DOI, there is no desensitization of the oxytocin or ACTH responses to 8-OH-DPAT even if one would subtract from the ACTH response the effect of DOI on baseline ACTH levels (Fig. 1). If the pretreatment with DOI would have depleted oxytocin, CRF, or ACTH stores, there should have been a reduced response to 8-OH-DPAT at 1 hr after DOI treatment. In addition, DOI pretreatment did not reduce the maximal hormone responses to 8-OH-DPAT challenge (Fig. 3). This indicates that DOI pretreatment has not depleted hormone stores. Thus DOI-induced attenuation of hormone responses to the 8-OH-DPAT challenge is more likely to be attributable to a heterologous desensitization of hypothalamic 5-HT$_{1A}$ receptors.

The hypothalamic paraventricular nucleus is crucial for the serotonergic stimulation of ACTH and oxytocin release (Liposits et al., 1987; Saphier, 1991; Kawano et al., 1992; Bagdy

and Makara, 1994; Rittenhouse et al., 1994; Van de Kar et al., 1995). Autoradiographic studies indicate a substantial density of [^{3}H]8-OH-DPAT-labeled 5-HT_{1A} receptors in the medial parvocellular divisions (containing CRF neurons) and the ventrolateral magnocellular divisions (containing oxytocin neurons) of the hypothalamic paraventricular nucleus (Li et al., 1997). The mRNA coding for 5-HT_{1A} receptors also has been detected in the paraventricular nucleus (Wright et al., 1995). Moreover, the pertussis toxin-resistant G_z-protein and G_z mRNA are expressed in the hypothalamic paraventricular nucleus (Serres et al., 2000). The 5-HT_{1A} receptor-mediated release of oxytocin and ACTH is not inhibited by pertussis toxin, but it is inhibited by G_z antisense oligodeoxynucleotides that reduce the level of G_z-protein in the paraventricular nucleus (Serres et al., 2000). Accordingly, 8-OH-DPAT increases the secretion of oxytocin and ACTH by activating 5-HT_{1A} receptor–G_z-protein signaling systems in the hypothalamic paraventricular nucleus.

Mechanical destruction of the hypothalamic paraventricular nucleus prevents the DOI-induced increase in plasma levels of oxytocin and ACTH (Bagdy, 1996). Autoradiographic studies indicate the presence of 5-HT_{2A} receptors in the hypothalamic paraventricular nucleus (Appel et al., 1990). In addition, *in situ* hybridization data indicate mRNA coding for 5-HT_{2A} receptors in the hypothalamic paraventricular nucleus (Wright et al., 1995; Gundlah et al., 1999). Activation of 5-HT_{2A} receptors with DOI increases Fos expression in oxytocin and CRF-expressing neurons, an effect that is blocked by the 5-HT_{2A} antagonist MDL 100,907 (Van de Kar et al., 2001). Combined, it is likely that 5-HT_{1A} receptors and 5-HT_{2A} receptors are colocalized on CRF and oxytocin cells, mediating oxytocin and ACTH release. Therefore, DOI-induced desensitization of the oxytocin and ACTH responses to a challenge with 8-OH-DPAT may represent a heterologous desensitization of 5-HT_{1A} receptor–G_z-protein signaling by activation of the 5-HT_{2A} receptors. However, there is no evidence showing that 5-HT_{1A} receptors or 5-HT_{2A} receptors are coexpressed in oxytocin or CRF cells, nor is there evidence indicating that 5-HT_{1A} receptors and 5-HT_{2A} receptors are colocalized in the same cells in the paraventricular hypothalamic nucleus. Thus an alternative explanation is that DOI may activate 5-HT_{2A} receptors on interneurons in a complex neuronal circuit, resulting in a desensitization of hypothalamic 5-HT_{1A} receptors.

The lack of reduction in the maximal response to 8-OH-DPAT after treatment with DOI suggests no downregulation of 5-HT_{1A} receptors. Consistent with this conclusion is the observation that [^{3}H]8-OH-DPAT binding to hypothalamic tissues was not reduced by DOI. The time interval after DOI treatment (2 hr) might be too short for a reduction in the density of 5-HT_{1A} receptors.

DOI-induced desensitization of hypothalamic 5-HT_{1A} receptor–G_z-protein signaling is characterized by delayed onset and short duration. These characteristics support a hypothesis of post-translational modification mechanisms such as phosphorylation, myristoylation, or palmitoylation (Hallak et al., 1994; Morales et al., 1998). Myristoylation and/or palmitoylation provide anchorage for G_z-proteins to associate with the plasma membrane and transmit extracellular signals through the receptor down the intracellular signal transduction cascade (Hallak et al., 1994; Beck et al., 1997). Because no change in membrane-associated G_z-proteins was observed, it is not likely that a change of the palmitoylation or myristoylation state of G_z-proteins is involved in DOI-induced desensitization of 5-HT_{1A} receptors. Phosphorylation plays an important role in short-term receptor desensitization (Chuang et al., 1996; Freedman and Lefkowitz, 1996). Cell culture studies demonstrate that activation of protein kinase C induces phosphorylation of 5-HT_{1A} receptors and desensitizes 5-HT_{1A} receptor-mediated inhibition of forskolin-stimulated cAMP accumulation (Raymond, 1991; Evans et al., 2001). Phosphorylation of G_z-proteins by protein kinase C prolongs the time during which $G_z\alpha$ stays in its uncoupled monomeric state (Fields and Casey, 1995) and reduces the ability of RGSZ1 proteins to potentiate the GTPase activity of $G_z\alpha$-proteins (Glick et al., 1998; Wang et al., 1998). Moreover, DOI has been reported to increase protein kinase C activity in several brain regions (Wang and Friedman, 1990; Rahimian and Hrdina, 1995). Therefore, it is possible that DOI activates 5-HT_{2A} receptors to increase the activity of protein kinase C, leading to phosphorylation of 5-HT_{1A} receptors and/or G_z-proteins and to desensitization of 5-HT_{1A} receptors in the hypothalamus.

DOI treatment does not reduce the coupling of 5-HT_{1A} receptors to G-proteins in the hypothalamus. This conclusion is supported by the observation that DOI did not reduce the binding of [^{3}H]8-OH-DPAT to 5-HT_{1A} receptors at a concentration close to its EC_{50} (0.8 nM). In addition, GTPγS-induced inhibition of [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors was not altered by DOI. However, 5-HT_{1A} receptors also have a high affinity for G_{i1}-, G_{i2}-, G_{i3}-, and G_o-proteins (Butkerait et al., 1995; Albert et al., 1996). GTPγS uncouples all $G_{i/o}$-proteins from 5-HT_{1A} receptors with no preference for G_z. Thus it is possible that an uncoupling of 5-HT_{1A} receptors from G_z-proteins in the hypothalamic paraventricular nucleus could be masked by a lack of change in G-protein coupling in other hypothalamic nuclei. Similarly, we measured only the level of membrane-associated G_z-proteins in the whole hypothalamus and could not rule out the possibility that DOI may alter G_z-proteins in the hypothalamic paraventricular nucleus.

In conclusion, the activation of 5-HT_{2A} receptors with DOI induces a delayed onset and transient functional heterologous desensitization of 5-HT_{1A} receptor signaling that is not attributable to a change in the coupling of 5-HT_{1A} receptors to G-proteins and is not attributable to a change in the level of membrane-associated G_z-proteins in the hypothalamus. This desensitization might be attributable to changes in signaling components distal to 5-HT_{1A} receptor–G_z-protein interaction.

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