Chronic Fluoxetine Differentially Affects 5-Hydroxytryptamine$_{2A}$ Receptor Signaling in Frontal Cortex, Oxytocin- and Corticotropin-Releasing Factor-Containing Neurons in Rat Paraventricular Nucleus


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ABSTRACT

Differential adaptive changes in serotonin$_{2A}$ [5-hydroxytryptamine (5-HT)$_{2A}$] receptor signaling during treatment may be one mechanism involved in the latency of therapeutic improvement with antidepressants, such as fluoxetine. We examined the effects of fluoxetine (2, 3, 7, 21, or 42 days) on hypothalamic 5-HT$_{2A}$ receptor signaling. The hormone responses to an injection of the 5-HT$_{2A}$ receptor agonist (1-H1006)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane HCl (DOI) were used as an index of hypothalamic 5-HT$_{2A}$ receptor function. Treatment with fluoxetine for 21 or 42 days produced diminished adrenocorticotropic hormone (ACTH) and oxytocin (but not corticosterone) responses to DOI injections (2.5 mg/kg i.p.; 15 min postinjection). Regulators of G protein signaling 4 and G$_{q/11}$ protein levels in the hypothalamic paraventricular nucleus were not altered during fluoxetine treatment. Because previous studies indicate that treatment with fluoxetine for 21 days resulted in increased hormone responses to DOI when measured at 30 min after injection, we examined the effect of fluoxetine (21 days) on DOI-induced increase hormone levels at 15, 30, and 60 min after DOI injection. Fluoxetine decreased the oxytocin response at 15 but not at 30 min post-DOI injection, and potentiated the ACTH and corticosterone responses at 30 min post-DOI injection. For comparison, we examined the effect of fluoxetine on 5-HT$_{2A}$ receptor-mediated increase in phospholipase C (PLC) activity in the frontal cortex. 5-HT-stimulated, but not guanosine 5'-O-(3-thio)triphosphate-stimulated PLC activity was increased after 21 days of fluoxetine-treatment. Overall, these results indicate that chronic fluoxetine treatment can potentiate 5-HT$_{2A}$ receptor signaling in frontal cortex but differentially alters 5-HT$_{2A}$ receptor signaling in oxytocin-containing neurons and corticotropin-releasing factor-containing neurons in the paraventricular nucleus.

Depression and other mood disorders associated with serotonergic dysfunction, including obsessive-compulsive disorder, premenstrual disorder, and anxiety are commonly treated with selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (Hollander et al., 1991; Steiner et al., 1995). A problem with SSRIs, as with all antidepressants, is that there is a 2 to 3 week lag time before clinical improvement is apparent (Nierenberg et al., 2000). This latency may be due to drug-induced changes in serotonin receptors and their signaling pathways. SSRIs produce long-term neuroadaptive changes in the serotonin 2 (5-HT$_2$) receptors (Cadogan et al., 1993; Li et al., 1993; Tilakaratne et al., 1995; Massou et al., 1997). Understanding these adaptive changes that occur in 5-HT$_{2A}$ receptor signaling may lead to novel antidepressants with a shorter latency of therapeutic action. In this study, we examined 5-HT$_{2A}$ receptor sensitivity and components of the signal transduction system during the time course of fluoxetine treatment from 2 to 42 days. Previous investigations have shown that treatment with fluoxetine for 21 days results in a supersensitivity of 5-HT$_{2A}$ receptor signaling in the cortex and hypothalamus of rats and in cortex of guinea pigs (Cadogan et al., 1993; Li et al., 1993; Tilakaratne et al., 1995). In the frontal cortex, 5-HT$_{2A}$ receptors are coupled to G$_{q/11}$ proteins (Roth et al., 1998), which in turn activate phospholipase C (PLC). PLC catalyzes

ABBREVIATIONS: SSRIs, selective serotonin reuptake inhibitor; 5-HT, 5-hydroxytryptamine; PLC, phospholipase C; GTP$_{Y}$S, guanosine 5'-O-(3-thio)triphosphate; DOI, (1-H1006)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane HCl; ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; MDL 100,907, (1-H1006)-2,3-dimethoxyphenyl-1-[2-4-(piperidine)-methanol]; RGS, regulators of G protein signaling; IOD, integrated optical density; ANOVA, analysis of variance.
the hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate. We measured PLC activity in the frontal cortex by monitoring its stimulation by 5-HT. Activation of PLC by 5-HT represents the overall activation of the signaling system, from 5-HT2A receptor, through Gq/11 protein to PLC. On the other hand, GTPγS activates PLC via G proteins without involvement of the 5-HT2A receptor. In our current study, 5-HT-mediated PLC activity was used to examine 5-HT2A receptor responsivity in the frontal cortex.

Hormone responses to an injection of the 5-HT2A receptor agonist DOI were used to examine 5-HT2A receptor responsivity in the hypothalamus. Plasma hormone concentrations are known to be regulated by neurons in the hypothalamus (Van de Kar, 1991). ACTH is secreted from the anterior pituitary gland, under hypothalamic control by corticotropin-releasing factor (CRF) secreted from parvocellular cells in the paraventricular nucleus. ACTH then activates the secretion of corticosterone from the adrenal gland. Oxytocin is secreted from nerve terminals located in the posterior lobe of the pituitary gland. The cell bodies that synthesize oxytocin are located in the hypothalamic supraoptic nucleus and magnocellular cells in the paraventricular nucleus.

DOI is a selective 5-HT2A receptor agonist (5-HT2A receptor, pKd = 8.89; 5-HT2C receptor, pKd = 8.19) with a low affinity for the other 5-HT receptor subtypes (5-HT1A receptor, pKd = 5.16; 5-HT1B receptor, pKd = 5.69) (Van Wijngaarden et al., 1990; Zifa and Fillion, 1992). Administration of DOI produces a reliable increase in the secretion of ACTH, corticosterone, and oxytocin through activation of 5-HT2A receptors. Several 5-HT2 receptor antagonists inhibit the effects of DOI on plasma hormone levels (Van de Kar, 1991). In the case of corticosterone, DOI can induce increases in its levels by both central and peripheral mechanisms (Alper, 1990; Rittenhouse et al., 1991). Recent evidence indicates that the effect of DOI on plasma levels of ACTH, corticosterone, oxytocin, prolactin, and renin is mediated by 5-HT2A receptors in the paraventricular nucleus of the hypothalamus, because intraparaventricular injections of the 5-HT2A receptor antagonist MDL 100,907 dose dependently blocked the DOI-induced increase in hormone levels (Zhang et al., 2002). Thus, the measurement of plasma ACTH and oxytocin after a peripheral injection of DOI is a useful marker of the functioning of 5-HT2A receptors in the paraventricular hypothalamic nucleus.

Regulators of G protein signaling (RGS) 4 proteins are involved in modulating the 5-HT2A receptor signaling cascade (Berman et al., 1996). RGS proteins potentiate the native ability of the Go subunit of G proteins to hydrolyze the GTP bound to it and thereby cause a reduction in the activation of the second messenger system. A decrease in RGS4 protein levels could induce a supersensitization response, whereas an increase in RGS4 protein levels could induce desensitization of 5-HT2A receptor signaling. We determined whether the levels of RGS4 protein in the paraventricular nucleus of the hypothalamus are altered at any time throughout chronic fluoxetine treatment. The levels of Goq protein also were measured in the paraventricular nucleus of the hypothalamus to determine a possible molecular mechanism underlying adaptive changes in 5-HT2A receptor signaling induced by chronic fluoxetine treatment. An increased amount of Goq protein would be expected to be associated with a potentiation of the hormone response.

Materials and Methods

Animals

Male Sprague-Dawley rats (225–250 g) were purchased from Harlan (Indianapolis, IN). The animals were housed two per cage in an environment controlled for lighting (7:00 AM–7:00 PM), temperature, and humidity. Food and water were available ad libitum. Eight to 12 rats were used per experimental group (12 being used in the saline-challenged groups). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the Loyola University Institutional Animal Care and Use Committee.

Drugs

Fluoxetine was donated by Eli Lilly & Co. (Indianapolis, IN). DOI [(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl] was purchased from Sigma/RBI (Natick, MA). Both drugs were dissolved in 0.9% saline. DOI was injected at doses of 0.5, 2.5, 5.0 mg/kg i.p. or at a dose of 2.5 mg/kg s.c. Fluoxetine was injected at a dose of 10 mg/kg/day i.p.

Experimental Procedures

All rats were handled for at least 7 days before the challenge injection and acclimated to a quiet environment to minimize stress. Rats were randomly assigned to the various experimental groups and cage mates received the same drug treatment. Weights were recorded every other day throughout the entire experiment with the final weight being taken the evening before the challenge injections.

Time Course of Fluoxetine Treatment. Fluoxetine (10 mg/kg/day i.p.) was injected daily for 2, 3, 7, 21, or 42 days. The initiation of these injections was staggered so that the animals were all given the challenge injection of DOI on the same day. Saline treatment (2 ml/kg i.p.) consisted of one group injected daily for 42 days. Challenge administration of DOI (0.5, 2.5, or 5.0 mg/kg i.p.) or saline (2 ml/kg i.p.) occurred 1 day after the last fluoxetine injection and 15 min before decapitation. The trunk blood was collected in centrifuge tubes containing 0.5 ml of a 0.3 M EDTA (pH 7.4) solution. The plasma samples were stored at –80°C until used for radioimmunoassays. Brains were removed, frozen on dry ice, and stored at –80°C until used for Western blot analyses.

Effect of Fluoxetine Treatment on the Time Course of Hormone Responses to DOI. Fluoxetine (10 mg/kg/day) or saline (2 ml/kg) was injected daily for 21 days. One day after the last fluoxetine injection, DOI (2.5 mg/kg or saline 2 ml/kg) was administered either by intraperitoneal injection 15, 30, or 60 min before decapitation or by subcutaneous injection 15 or 30 min before decapitation. Control rats received saline injection. The trunk blood was collected and plasma was stored as mentioned previously.

Radioimmunoassay of Hormones. Plasma ACTH, corticosterone (Li et al., 1993), and oxytocin (Li et al., 1997a) concentrations were determined by radioimmunoassays as described previously. Intra-assay variability is 4.2% for ACTH, 4.5% for corticosterone, and 5% for oxytocin. Interassay variability is 14.6% for ACTH, 11.9% for corticosterone, and 9% for oxytocin.

Immunoblot Analysis of Goq and RGS4 Proteins

Tissue Preparation. The hypothalamic paraventricular nuclei from the 0.5 mg/kg DOI challenge group across all the various fluoxetine treatments were used for RGS4 and Goq protein measurements. The paraventricular nuclei used in these analyses were dissected from a 700-μm coronal section obtained using a cryostat (–10°C). Punches of paraventricular nuclei were sonicated in 100 μl of 10 mM Tris buffer containing 0.1 M NaCl, 0.1 M EDTA, and protease inhibitor cocktail (1:1000) from Sigma-Aldrich (St. Louis,
MO). The frontal cortex from the saline-challenged group receiving 21-day fluoxetine and 21-day saline treatment was used for RGS4 and Gq protein measurements. The tissue was prepared as described previously (Wolf and Schutz, 1997). Briefly, 3 μg/μl membrane homogenates of frontal cortex were diluted into 100 μl of total volume. The reaction was carried out in 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, 300 nM free Ca2+, 3 μM 5-HT, and 1.1 mM unlabeled phosphatidylinositol. The assay tubes were kept on ice until the incubation was started by addition of 100 μM [3H]phosphatidylinositol. The tubes were subsequently incubated for 20 min at 37°C. The reaction was stopped by adding 0.9 ml of CHCl2/MeOH (1:2) and 0.3 ml of chloroform. The assay tubes were shaken vigorously for 90 s and centrifuged at 9000 rpm at room temperature for 90 s. Then 0.3 ml of the upper aqueous phase was mixed with 6 ml of scintillation cocktail and counted on a scintillation counter for 7 min.

Statistical Analyses

All data are represented as group mean ± S.E.M. Body weights were analyzed were analyzed using a one-way analyses of variance (ANOVA) and two-way repeated measures ANOVA followed by a Newman-Keuls post hoc analysis. Hormone data from the fluoxetine time-course experiment were analyzed by two-way ANOVA, whereas the hormone data from the DOI challenge time-course experiments were analyzed by one-way ANOVA. Newman-Keuls post hoc analysis was used as a follow-up test for all hormone data. Gq and RGS4 protein levels were analyzed using a one-way ANOVA for the fluoxetine time-course and a Student’s t test for the 21-day fluoxetine treatment. PLC activity was analyzed by a Student’s t test. GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD) was used for all statistical analyses.

Results

Body Weight

All rats showed a steady increase in body weight during the drug treatment period. Chronic fluoxetine (10 mg/kg i.p.) significantly inhibited this growth rate during the 42 days of the experiment compared with their saline (2 ml/kg i.p.-) injected controls as shown in Fig. 1A [F(1,1422) = 710.66, p < 0.0001]. In rats treated for 42 days, the difference in mean daily weight between the fluoxetine-treated group and the saline-treated group was significant within 8 days (Fig. 1A) [F(1,1422) = 5.77, p < 0.0001]. The magnitude of this difference in body weight increased as the duration of fluoxetine injections increased.

The mean body weights on the final day of the fluoxetine or saline injections for each treatment group (i.e., rats treated with fluoxetine for 2, 3, 7, 21, and 42 days) are depicted in Fig. 1B. The difference in the final body weight was dependent on the duration of fluoxetine injection [F(3,210) = 18.20, p < 0.001]. The body weights were significantly different in the rats treated with fluoxetine for 7 days or longer (7, 21, and 42 days) compared with saline controls.

Plasma Hormone Levels

Time Course of Fluoxetine. Injection of DOI (2.5 and 5.0 mg/kg) significantly increased the plasma levels of ACTH [F(3,193) = 94.46, p < 0.0001] and oxytocin [F(3,170) = 121.09, p < 0.0001] in the saline-treated group (Fig. 2, A-C, white columns). Fluoxetine treatment for 21 or 42 days inhibited the effect of DOI on ACTH [F(15,170) = 2.75, p < 0.001] and oxytocin [F(15,170) = 4.08, p < 0.0001] levels (Fig. 2, A and C). This desensitization was evident at the 2.5-mg/kg dose of DOI for ACTH and at the 2.5- and 5.0-mg/kg doses for oxytocin. Fluoxetine treatment for 3 and 7 days also reduced the effect of the 5.0 mg/kg dose of DOI on plasma oxytocin levels. No change was seen in the levels of corticosterone during fluoxetine treatment (Fig. 2B) [F(15,169) = 1.58, p = 0.083].

Effect of Fluoxetine (21 Days) on Hormone Time-Course Responses to DOI (i.p.). Rats received daily injections of saline or fluoxetine (10 mg/kg/day i.p.) for 21 days. Administration of DOI (2.5 mg/kg i.p.) to saline-pretreated rats produced a significant increase in plasma ACTH [F(9,64) = 18.49, p < 0.0001], corticosterone [F(9,65) = 42.36, p < 0.0001] and oxytocin [F(9,64) = 19.93, p < 0.0001] levels 15,
30, and 60 min postinjection. The DOI-induced increase in plasma ACTH and oxytocin levels was maximal at 15 min postinjection. The oxytocin response to DOI was still maximal at 30 min postinjection and subsided by 60 min (Fig. 3C). The corticosterone response was more delayed and reached a maximum at 60 min postinjection (Fig. 3B).

Treatment with fluoxetine resulted in a reduced ACTH response at 15 min after injection of DOI, but a potentiation at 30 min post-DOI injection (Fig. 3A). At 60 min after DOI challenge, there were no significant differences between fluoxetine- and saline-treated rats. Fluoxetine potentiated the effect of DOI on plasma corticosterone at 30 min after DOI injection, but did not potentiate the effect of DOI at 15 or 60 min after injection (Fig. 3B). Treatment with fluoxetine for 21 days resulted in a reduced oxytocin response at 15 min after DOI injection, but the oxytocin response to DOI at 30 min postinjection was not different from the response in saline-treated rats (Fig. 3C).

Effect of Fluoxetine (21 Days) on Hormone Time-Course Responses to DOI (s.c.). Hormone responses to subcutaneous DOI injections in saline- and fluoxetine-treated rats were examined to determine whether the route of administration of DOI impacts on the hormone responses. Administration of DOI (2.5 mg/kg s.c.) produced a significant increase in plasma ACTH \( [F_{5,39} = 21.38, p < 0.0001] \), corticosterone \( [F_{5,39} = \)
Fig. 3. Time course of hormone responses to intraperitoneal DOI (2.5 mg/kg i.p.) (15-, 30-, and 60-min challenge intervals) after 21 days of saline (2 ml/kg) or fluoxetine (10 mg/kg/day i.p.) treatment. The data represent the mean ± S.E.M. of ACTH (A), corticosterone (B), and oxytocin (C) levels (n = 6–8). A significant effect of fluoxetine compared with saline treatment is indicated by *, p < 0.05 and **, p < 0.01. A significant effect of DOI is indicated by †, p < 0.05 and ††, p < 0.01 (one-way ANOVA and Newman-Keuls’ multiple range test).

Fig. 4. Time course of hormone responses to subcutaneous DOI (2.5 mg/kg) (15- and 30-min challenge intervals) after 21 days of fluoxetine (10 mg/kg/day i.p.) or saline (2 ml/kg i.p.) treatment. The data represent the mean ± S.E.M. of ACTH (A), corticosterone (B), and oxytocin (C) levels (n = 6–8). A significant effect of fluoxetine compared with saline treatment is indicated by *, p < 0.05 and **, p < 0.01. A significant effect of DOI is indicated by †, p < 0.05 and ††, p < 0.01 (one-way ANOVA and Newman-Keuls multiple range test).
27.27, \( p < 0.0001 \), and oxytocin \( [F_{(5,39)} = 12.38, p < 0.0001] \) levels both at 15 and 30 min postinjection (Fig. 4). These results in saline-pretreated rats, with s.c. injections of DOI, are similar to those seen with the i.p. administration of DOI. Fluoxetine treatment produced no desensitization of the ACTH response to DOI at 15 min postinjection, but a potentiated ACTH response at 30 min after subcutaneous injections of DOI \( (p < 0.05 \) by Newman-Keuls test; Fig. 4A). Note that the potentiation of the ACTH response to DOI was observed at 30 min post-DOI injection with both routes of DOI administration (i.p. and s.c.) (Figs. 3A and 4A). Consistent with the previous experiment, fluoxetine treatment potentiated the corticosterone response to DOI when measured at 30 min post-DOI injection \( (p < 0.05 \) by Newman-Keuls test; Fig. 4B). Fluoxetine treatment reduced the oxytocin response 15 min after a DOI injection \( (p < 0.05 \) by Newman-Keuls test; Fig. 4C). However, the oxytocin response at 30 min after DOI injection was not different from the oxytocin response in the saline pretreatment controls (Fig. 4C). These hormone responses in the fluoxetine-treated rats given subcutaneous injections of DOI are similar to those seen in fluoxetine-treated rats given intraperitoneal injections of DOI.

**Gq and RGS4 Protein Levels**

Chronic treatment with fluoxetine did not alter the levels of \( G_q \) or RGS4 proteins in the "punches" of the hypothalamic paraventricular nucleus or in the frontal cortex. The one-way ANOVA indicated no significant difference in RGS4 \( [F_{(5,42)} = 0.175, p > 0.05] \) or \( G_q \) protein \( [F_{(5,42)} = 0.573, p > 0.05] \) concentrations across the time periods of treatment with fluoxetine, including 2, 3, 7, 21 and 42 days of injections in the hypothalamic paraventricular nucleus (Fig. 5, B and C). When comparing 21-day fluoxetine treatment against the 21-day saline treatment, the Student's \( t \) test indicated no significant difference in RGS4 \( (p = 0.52) \) or \( G_q \) protein \( (p = 0.14) \) levels in the frontal cortex. We found no difference in RGS4 protein levels after DOI challenge compared with saline-challenged rats (data not shown).

**PLC Activity**

Chronic fluoxetine treatment (21 days) potentiated the 5-HT-induced stimulation of PLC activity in the frontal cortex (Fig. 6A). 5-HT-stimulated PLC activity was increased by 18.9% after 21 days of fluoxetine injections \( (p < 0.05) \). GTP\(\gamma S\)-stimulated PLC activity in the frontal cortex (representing direct stimulation of the enzyme by G proteins) was not altered after treatment with fluoxetine \( (p = 0.88) \) (Fig. 6B). It was not possible to measure 5-HT-stimulated PLC activity in the punches of the hypothalamic paraventricular nucleus due to the small amount of tissue.

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**Fig. 5.** A. representative Western blots of \( G_q \) and RGS4 proteins in paraventricular nuclei of rats treated with saline (0) or fluoxetine for 2, 3, 7, 21, and 42 days. Actin was used to verify the loading of protein in each lane. The mean ± S.E.M. of \( G_q \) protein (B) and RGS4 protein (C) levels in the hypothalamic paraventricular nucleus (PVN) and \( G_q \) protein (D) and RGS4 protein (E) in frontal cortex indicate that fluoxetine treatment produced no change in the levels of \( G_q \) or RGS4 proteins \( (n = 6) \).
Fluoxetine Differentially Affects 5-HT<sub>2A</sub> Receptor Signaling

The influence of treatment with fluoxetine on hormone responses to DOI ranged from attenuation of the oxytocin response (at 15 min post-DOI injection), to potentiation for ACTH and corticosterone responses at 30 min after DOI injection. A summary of the impact of fluoxetine treatment on hormone secretion mediated by 5-HT<sub>2A</sub> receptors is shown in Table 1. Fluoxetine treatment also produced a decrease in the rate of weight gain. Additionally, our neurochemical data indicate that treatment with fluoxetine also produces a supersensitization of 5-HT<sub>2A</sub> receptors in the frontal cortex. Fluoxetine produced a decrease in the rate of weight gain but not a decrease in body weight. This phenomenon in rats has been published previously (Alper, 1992). Fluoxetine has also successfully been used in humans as a treatment for obesity (Goldstein et al., 1994). Fluoxetine has an anorectic effect that may lead to this decrease in weight gain observed clinically and experimentally (McGuirk and Silverstone, 1990). Fluoxetine-induced hypophagia is thought to be mediated by fluoxetine acting on the 5-HT<sub>2C</sub> receptor (Lightowler et al., 1996). The 5-HT<sub>2C</sub> receptor is important in feeding as 5-HT<sub>2C</sub> receptor knockout mice have increased feeding and obesity (Tecott et al., 1995).

Stimulation of 5-HT<sub>2A</sub> receptors increases the secretion of ACTH, corticosterone, and oxytocin and increases Fos expression in CRF and oxytocin neurons in the paraventricular nucleus of the hypothalamus (Van de Kar, 1991). 5-HT<sub>2A</sub> receptors in the hypothalamic paraventricular nucleus mediate the DOI-stimulated increases in plasma ACTH and oxytocin levels, because intraparaventricular and peripheral injections of the 5-HT<sub>2A</sub> receptor antagonist MDL 100,907 dose dependently inhibit these responses (Van de Kar et al., 2001; Zhang et al., 2002). Hence, the magnitude of DOI-induced increases in plasma ACTH and oxytocin levels can be used as a peripheral marker of 5-HT<sub>2A</sub> receptor function in the hypothalamic paraventricular nucleus.

The oxytocin data are probably the most puzzling. We have now repeated this experiment twice, once using a DOI challenge administered intraperitoneally and once by subcutaneous administration. In both experiments, treatment with fluoxetine resulted in an attenuation of the oxytocin response when examined 15 min after DOI injection and no change in the oxytocin response at 30 min after DOI injection. Hence, the data suggest that treatment with fluoxetine produces either a delayed onset or a desensitization of 5-HT<sub>2A</sub> receptor signaling in oxytocin-expressing neurons. On the other hand, treatment with fluoxetine resulted in potentiation of the effect of DOI on both ACTH and corticosterone levels, suggesting an increase in 5-HT<sub>2A</sub> receptor signaling in CRF neurons. However, the reduced ACTH response to DOI at 15 min post-DOI injection suggests a delay in the onset of receptor signaling of supersensitive 5-HT<sub>2A</sub> receptors. Although previous in vitro studies using human embryonic kidney-293 and C6 glioma cells reported cell type-specific 5-HT<sub>2A</sub> receptor regulation (Gray et al., 2001), to our knowledge, this is the first demonstration of such a phenomenon in vivo.

Although treatment with fluoxetine produces a delay in both the ACTH and oxytocin responses to DOI, fluoxetine may cause desensitization in 5-HT<sub>2A</sub> signaling in the oxytocin neurons and a supersensitive response in CRF neurons in the paraventricular nucleus. The differences in fluoxetine-induced changes between the ACTH and oxytocin responses to DOI suggest that 5-HT<sub>2A</sub> receptors in the oxytocin-con-
taining magnocellular cells and the CRF-containing parvo-
cellular cells in the paraventricular nucleus are regulated
differently.

A second possible reason for the differences in the time
course of hormone responses to DOI is its route of adminis-
tration and subsequent differences in the rate of absorption
of DOI from the site of administration. Fluoxetine was noted
to produce tissue necrosis at the site of administration (Fish-
er et al., 1999) and indeed tissue necrosis was seen in the
peritoneal cavities of rats injected with fluoxetine for 21 days
in our studies. Tissue necrosis could result in less efficient
absorption of DOI and thus explain the attenuation of hor-
mone levels at the 15-min time point compared with saline
controls. However, a subcutaneous injection of DOI, which
avoided the peritoneum would not have a problem of de-
creased absorption of DOI. Yet the same phenomenon of
delayed hormone response, after fluoxetine treatment, was
observed when DOI was injected subcutaneously, suggesting
that the delayed oxytocin and ACTH responses are not due to
differences in DOI absorption from the site of injection.

Treatment with fluoxetine resulted in a shift in the ACTH
time course to the right, thereby $E_{\text{max}}$ is reached at 30 min
rather than 15 min post-DOI injection. In contrast to ACTH,
treatment with fluoxetine shifts the time course for cortico-
sterone to the left. Thus, $E_{\text{max}}$ is reached at 30 min rather
than 60 min post-DOI challenge injection. If corticosterone
secretion were solely dependent on central serotoninergic
control, then we would expect to see the same rightward shift
of the corticosterone time course as is seen with the ACTH
time course. Plasma corticosterone levels are also regulated
by peripheral serotoninergic mechanisms (Alper, 1990), which
may help to explain why corticosterone is undergoing an
opposite shift in its time course compared with ACTH.

The analysis of 5-HT-stimulated PLC activity in the fron-
tal cortex confirms the conclusion that treatment with fluox-
etine can lead to supersensitive 5-HT$_{2A}$ receptors. Our anal-
ysis compared the activation of PLC with 5-HT to activation
with GTP$_S$, a nonhydrolyzable form of GTP, which irrevers-
ibly activates G proteins. The determination of 5-HT-stimu-
lated PLC activity examines the functioning of 5-HT$_{2A}$ recep-
tor signaling from the receptor, through the G protein to the
effector enzyme (PLC). On the other hand, GTP$_S$ directly
binds to $G_{\text{q/11}}$ proteins, changing their confirmation. Acti-
vated GTP$_S$-G$_{\text{q/11}}$ proteins then directly activate PLC.
5-HT-stimulated PLC activity is 5-HT$_{2A}$ receptor mediated in
the frontal cortex because the 5-HT$_{2A}$ receptor antagonists
ketanserin, spiperone, and mianserin significantly attenuate
the 5-HT-stimulated PLC activity (Wolf and Schutz, 1997).
Fluoxetine treatment (21 days) increased 5-HT-stimulated
PLC activity without changing GTP$_S$-stimulated PLC ac-
tivity. This not only shows a supersensitivity of the 5-HT$_{2A}$
receptors at the cellular level in the frontal cortex, but also
shows that the supersensitivity involves a change between
5-HT$_{2A}$ receptors and $G_{\text{q/11}}$ proteins and not between $G_{\text{q/11}}$
proteins and the effector enzyme PLC.

This supersensitivity of 5-HT$_{2A}$ receptors in the frontal
cortex is observed at a cellular level with only one level of
amplification (G proteins) between receptor stimulation and
effector activation. Unfortunately, the hypothalamic para-
ventricular nucleus does not provide sufficient tissue to de-
termine PLC activity. But because supersensitivity of 5-HT$_{2A}$
receptors occurs in both CRF neurons and frontal cortex
neurons after prolonged fluoxetine exposure, it is likely that
the mechanism of 5-HT$_{2A}$ receptor supersensitivity is similar
in both of these neuronal cell types.

Alterations in $G_{\text{q/11}}$ or RGS4 proteins could underlie
changes in sensitivity of 5-HT$_{2A}$ receptor signaling. However,
no changes were observed in the levels of $G_{\text{q/11}}$ or RGS4 pro-
teins in the paraventricular nucleus at the time periods of
fluoxetine treatment (2, 3, 7, 21, or 42 days) or in the frontal
cortex after 21 days of fluoxetine treatment. It is possible
that differential regulation of 5-HT$_{2A}$ receptor signaling in
the oxytocin neurons and CRF neurons in the paraventricu-
lar nucleus might obscure changes in the 5-HT$_{2A}$ receptor
signaling proteins in the whole punch of the paraventricular
nucleus. Just as PLC activity increased by approximately
20% in the frontal cortex after 21 days of fluoxetine treat-
ment, the mean in $G_{\text{q/11}}$ protein levels were 55% above control
levels. However, the changes in the levels of $G_{\text{q/11}}$ proteins
in the frontal cortex were not statistically significant and,
therefore, cannot explain the supersensitization of the 5-HT$_{2A}$
Receptor in the frontal cortex.

Treatment with fluoxetine for 7 or 14 days was accompa-
yed by an increase in high-affinity agonist $^{125}$I-DOI-labeled
5-HT$_{2A}$ receptor binding but no change in the density of
agonist $[^3H]$ketanserin-labeled 5-HT$_{2A}$ receptors, or $G_{\text{q/11}}$
and $G_{\text{q/11}}$ protein levels in the hypothalamus (Li et al.,
1997b). Receptor phosphorylation in other systems has been
shown to inhibit the coupling of G proteins to receptors
(Pitcher et al., 1998). For 5-HT$_{2A}$ receptors, rapid agonist-
duced desensitization was dependent on phosphorylation
by protein kinase C (Roth et al., 1986; Berg et al., 2001).
Thus, fluoxetine might induce alterations in posttransla-
tional modifications of 5-HT$_{2A}$ receptors or G proteins,
leading to changes in 5-HT$_{2A}$ receptor coupling to G proteins
rather than a change in the protein levels of the signaling
system.

In summary, our data suggest that sustained treatment
with fluoxetine produces a differential change in the sensi-
tivity of 5-HT$_{2A}$ receptor signaling in the frontal cortex and
CRF-containing neurons in the paraventricular nucleus com-
pared with oxytocin-containing neurons in the paraventricu-
lar nucleus. Fluoxetine treatment for 21 and 42 days in-
creases the sensitivity of 5-HT$_{2A}$ receptor-mediated
responses in the CRF-containing neurons of the hypothal-
amic paraventricular nucleus and in neurons in the frontal
cortex and decreases the sensitivity of 5-HT$_{2A}$ receptor-me-
diated responses in hypothalamic oxytocin-containing neu-
rons. These fluoxetine-induced changes in 5-HT$_{2A}$ receptor
signaling occur without changing the levels of $G_{\text{q/11}}$ or RGS4
proteins. In the frontal cortex, the mechanism is likely at the
level of the 5-HT$_{2A}$ receptor or receptor-G protein interaction.

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