

Sustained Desensitization of Hypothalamic 5-Hydroxytryptamine_{1A} Receptors after Discontinuation of Fluoxetine: Inhibited Neuroendocrine Responses to 8-Hydroxy-2-(Dipropylamino)Tetralin in the Absence of Changes in G_{i/o/z} Proteins¹

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ABSTRACT

Long-term exposure to fluoxetine produces a desensitization of hypothalamic postsynaptic 5-hydroxytryptamine (5-HT)_{1A} receptors, indicated by a substantial inhibition of the 5-HT_{1A} receptor-mediated stimulation of oxytocin and adrenocorticotrophic hormone (ACTH) secretion. The present study investigated the time course and mechanism of this desensitization after discontinuation of fluoxetine administration. Male rats were injected with saline or fluoxetine (10 mg/kg/day, i.p.) for 14 days and were challenged with a 5-HT_{1A} agonist, [8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) 50 µg/kg, s.c.] 2, 4, 7, 14, 28, or 60 days post-treatment. In control animals, 8-OH-DPAT significantly increased (approximately 15-fold) plasma levels of oxytocin and ACTH. At 2 days post-treatment, oxytocin and ACTH responses to 8-OH-DPAT were reduced by 74% and 68%, respectively. During further withdrawal from fluoxetine, there was a gradual increase in the oxytocin response

toward control levels. However, even 60 days after discontinuation of fluoxetine, the oxytocin response was still significantly reduced by 26% compared with controls. In contrast, the suppressed ACTH response to 8-OH-DPAT (a less-sensitive indicator of desensitization) gradually returned to control levels by day 14 of withdrawal from fluoxetine. Interestingly, the sustained reductions in the hormone responses occurred in the absence of reductions in G_z or G_i protein levels in the hypothalamus. Furthermore, this desensitization was sustained in the absence of detectable levels of fluoxetine and norfluoxetine in plasma and brain tissue. These findings suggest that the sustained desensitization of hypothalamic 5-HT_{1A} receptor systems, observed during fluoxetine withdrawal, may be due to altered interactions among the protein components of the 5-HT_{1A} receptor system, rather than their absolute levels.

Selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, are widely prescribed for the treatment of disorders associated with serotonergic dysfunction. Such disorders include depression, anxiety, obsessive-compulsive disorder, aggression, and bulimia (Fuller, 1996). Abrupt cessation of treatment with SSRIs, especially ones with a shorter half-life like paroxetine or fluvoxamine, can induce a characteristic cluster of somatic and psychological symptoms, including dizziness, gastrointestinal and sensory disturbances, fatigue, anxiety, and irritability (Coupland et al., 1996;

Schatzberg et al., 1997b; Zajecka et al., 1997). Another concern regarding the discontinuation of SSRIs is the inability to predict the likelihood of the recurrence of original symptoms once treatment has stopped.

Repeated administration of SSRIs induces desensitization of somatodendritic serotonin_{1A} [5-hydroxytryptamine (5-HT)_{1A}] autoreceptors in the raphe region (Le Poul et al., 1995) as well as a subsequent desensitization of the postsynaptic 5-HT_{1A} receptors in forebrain regions like the hypothalamus (Lesch et al., 1991; Li et al., 1993b, 1994, 1996, 1997; Lerer et al., 1997; Sargent et al., 1997). Measuring the hormone responses to 5-HT_{1A} receptor agonists in humans and in rats has provided an indicator of the function of postsynaptic 5-HT_{1A} receptor systems in the hypothalamus. For example, we previously reported that daily injections of 10 mg/kg fluoxetine or paroxetine for 14 to 21 days to rats

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); 8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin; ACTH, adrenocorticotrophic hormone; ANOVA, analysis of variance; CRH, corticotrophin-releasing hormone; HPLC, high-pressure liquid chromatography; IOD, integrated optical density; SSRI, selective serotonin reuptake inhibitor.

completely inhibits the oxytocin, adrenocorticotrophic hormone (ACTH), and corticosterone responses to the 5-HT_{1A} agonists 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) (Li et al., 1993b, 1996, 1997) or ipsapirone (Li et al., 1994) measured 18 h after the last fluoxetine injection. Similar observations have also been reported in humans (Lesch et al., 1991; Lerer et al., 1997; Sargent et al., 1997). These findings suggest that long-term exposure to 5-HT uptake blockers triggers adaptive mechanisms that induce the desensitization of hypothalamic 5-HT_{1A} receptors. Because symptomatic relief in patients is usually delayed for 2 to 3 weeks after initiation of treatment, it is likely that these adaptive changes are important in the therapeutic effects of SSRIs. It is unclear what role these changes have after the cessation of treatment.

The physiological basis of symptoms experienced after discontinuation of SSRIs in humans remains speculative. The sustained blockade of 5-HT reuptake during exposure to SSRIs produces a gradual increase in 5-HT levels in the synapse, producing a subsequent desensitization of postsynaptic 5-HT_{1A} receptors (Auerbach and Hjorth, 1995; Kreiss and Lucki, 1995). Once treatment is discontinued, it is possible that 5-HT reuptake activity returns to normal, causing an acute reduction of synaptic 5-HT levels in the presence of desensitized postsynaptic 5-HT_{1A} receptors. This subsequent hyposerotonergic state could alter the function of several other interacting neurotransmitter systems, resulting in a variety of clinical symptoms (Schatzberg et al., 1997a; Zajecka et al., 1997). Over time, there would be a gradual return of 5-HT release, corresponding to the gradual improvement of withdrawal symptoms in patients discontinued from SSRIs. The purpose of the present study was to determine how long the desensitization of the 5-HT_{1A} receptor system persists after the discontinuation of fluoxetine.

We showed previously that long-term exposure to fluoxetine in rats does not affect the density or the affinity of hypothalamic postsynaptic 5-HT_{1A} receptors (Li et al., 1993b), but produces a decline in hypothalamic levels of G_z, G₁₁, and G₁₃ proteins that parallels the decline in hormone responses to a 5-HT_{1A} agonist (Li et al., 1996; Raap et al., 1999). Hence, the desensitization of 5-HT_{1A} receptors likely involves receptor signaling mechanisms. The family of G_i proteins is composed of G₁₁, G₁₂, G₁₃, G_o, and G_z proteins and is known to couple 5-HT_{1A} receptors to effector mechanisms (Raymond et al., 1993; Barr et al., 1997). Our recent studies indicate that hypothalamic G_z proteins mediate the ACTH and oxytocin responses to activation of 5-HT_{1A} receptors (Serres et al., 1998; Van de Kar et al., 1998b). If the reduction in the G_z proteins underlies fluoxetine-induced desensitization of 5-HT_{1A} receptors, then both the neuroendocrine and G_z protein changes may also follow a similar time course during withdrawal from fluoxetine.

Because the half-life of fluoxetine and its active metabolite norfluoxetine are different in humans and in rats (Caccia et al., 1990; DeVane, 1994), it is also necessary to determine the relationship between the pharmacokinetics of fluoxetine and the changes induced by long-term exposure. In the present study, we measured the levels of fluoxetine and norfluoxetine and the neuroendocrine responses to 8-OH-DPAT through 60 days of withdrawal from fluoxetine to determine the relationship between brain and plasma levels of fluoxetine and norflu-

oxetine, and the biochemical manifestations of the desensitization of postsynaptic 5-HT_{1A} receptors in the hypothalamus.

Materials and Methods

Animals

Male Sprague-Dawley rats (225–250 g) were purchased from Harlan Laboratories (Indianapolis, IN). Animals were housed two per cage in a room controlled for temperature, humidity, and lighting (lights on 7 AM–7 PM). Food and water were available ad libitum at all times. 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 HCl was donated by Eli Lilly and Co. (Indianapolis, IN). A fresh fluoxetine solution was made daily by dissolving fluoxetine in the vehicle (0.9% saline) and injected (i.p.) in a volume of 2 ml/kg. (\pm)-8-OH-DPAT HBr was purchased from Research Biochemical Inc. (Natick, MA); it was dissolved in saline and injected (s.c.) in a volume of 1 ml/kg. The doses of both drugs were calculated as the salt complex.

Animal Drug Treatments

Conscious rats were injected (i.p.) once daily for 14 days with either saline or 10 mg/kg/day of fluoxetine and were sacrificed either 2, 4, 7, 14, 28, or 60 days after the last fluoxetine injection. Body weight was monitored throughout the injection and withdrawal periods. Rats were challenged with either saline or 8-OH-DPAT (50 μ g/kg, s.c.) 15 min before decapitation. Maximal stimulation of the secretion of oxytocin and ACTH is reached by 8-OH-DPAT doses of 200 to 500 μ g/kg (s.c.) (Li et al., 1993b) or even higher doses (Gilbert et al., 1988). A submaximal dose of 8-OH-DPAT was used in the present study (50 μ g/kg, s.c.) to prevent activation of other 5-HT receptor subtypes. This dose of 8-OH-DPAT was previously shown to be the most effective dose in demonstrating desensitization of 5-HT_{1A} receptors (Li et al., 1996). Trunk blood was collected in centrifuge tubes containing 0.5 ml of a 0.3 M EDTA (pH 7.4) solution. The plasma was separated and stored at -70°C for the measurement of hormones, fluoxetine, and norfluoxetine levels. The hypothalamus was removed from the brain immediately after decapitation and frozen in liquid nitrogen, and then stored at -70°C until the analysis of G protein levels.

Radioimmunoassay of Hormones

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

Immunoblots of G Proteins

Protein Fractionation. G protein levels were measured in hypothalamic tissue from animals that received a saline challenge before sacrifice. All procedures were conducted at 4°C unless otherwise indicated. Briefly, the tissues were homogenized in 0.5 ml of a 50 mM Tris buffer, pH 7.4 containing 150 mM NaCl, 10% sucrose, and 0.5 mM phenylmethanesulfonyl fluoride. After centrifugation at 20,000g for 60 min, the pellets (containing the membrane-bound proteins) were solubilized in a 20 mM Tris buffer, pH 8, containing 1 mM EDTA, 100 mM NaCl, 1% of sodium cholate, and 1 mM dithiothreitol in a ratio of 3 μ l of buffer/mg tissue. The resuspended homogenates were incubated and shaken for 1 h, followed by centrifugation at 100,000g for 60 min. The supernatant (containing the membrane-bound G proteins) was collected and stored for the determination of membrane-bound G protein levels. Protein concentrations were measured using bovine serum albumin as a standard. The total-protein concentrations were between 3.6 and 5.4 μ g/ μ l.

Quantification of G Proteins. The solubilized proteins were resolved by SDS-polyacrylamide gel electrophoresis with 0.75-mm thick Tris-glycine denaturing reducing gels, containing 0.1% SDS, 12.5% acrylamide/bisacrylamide (30:0.2), 4.6 M urea, and 375 mM Tris, pH 8.7. Three samples from each treatment group and three randomly selected controls were loaded on each gel. Each sample was measured on three independent gels. The proteins were then electrophoretically transferred for 2 h to nitrocellulose membranes, which were then allowed to dry. The membranes were incubated in a solution containing 5% nonfat dry milk, 0.05% Nonidet P-40, 50 mM Tris, and 150 mM NaCl, pH 7.4, for 1 h and were then washed. The membranes were incubated with polyclonal antisera for G_{11/2} (AS/7, 1:2500 dilution; DuPont NEN, Boston, MA), G₁₃ (Anti-G₁₃, 1:2000 dilution; Upstate Biotechnology Inc., Lake Placid, NY), G_o (GC/2, 1:10,000 dilution; DuPont NEN), and G_z (I-20, 1:6000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C overnight. The membranes were then incubated with a secondary antibody (goat anti-rabbit serum, 1:5000 dilution; Cappell, Organon Teknika Corp., Durham NC) for 60 min. Following four washes with 0.05% Nonidet P-40 in 50 mM Tris and 150 mM NaCl, the membranes were incubated with rabbit peroxidase-antiperoxidase (1:10,000; Cappell, Organon Teknika Corp.) for 1 h. The membranes were incubated with the enhanced chemiluminescence substrate solution (Amersham, Arlington Heights, IL) for 1 min and then exposed to Kodak X-ray film (Eastman Kodak, Rochester, NY) for 10 to 60 s.

Data Analysis. Films were analyzed densitometrically using NIH Image (v 1.57) for Macintosh computers. The gray scale density readings were calibrated using a transmission step wedge standard. The integrated optical density (IOD) of each band was calculated as the sum of optical densities of all the pixels within the area of the band outlined. An area adjacent to the G protein bands was used to calculate the background optical density of the film. The IOD for the film background was subtracted from the IOD for each band. The resulting IOD for each G protein band was then divided by the amount of protein loaded on the corresponding lane. The IOD per microgram protein values obtained from treated rats were divided by the mean IOD per microgram protein values obtained from control rats in each gel to determine the relative amounts of the G proteins. The data for each rat were the means obtained from the three gels.

High-Pressure Liquid Chromatography (HPLC) of Fluoxetine and Norfluoxetine

Plasma Preparation. Fluoxetine and norfluoxetine levels were measured in plasma from animals that received a saline challenge before sacrifice. Extraction of fluoxetine and norfluoxetine from plasma was carried out at room temperature. In a 2-ml polypropylene microfuge tube, 5 μ l of a 100 μ M fluvoxamine solution in 0.01 M H₃PO₄ (internal standard) was added to 500 μ l of thawed plasma. Five hundred microliters of 1 M NaOH was added and tubes were mixed. Six hundred microliters of chloroform was then added and tubes were shaken vigorously for 3 min. Tubes were then centrifuged at 9000g for 1 min, and the aqueous phase was aspirated and discarded. Compounds were back-extracted from the chloroform phase by adding 400 μ l of 0.1 M H₃PO₄, shaking for 3 min, and centrifuging at 9000g for 1 min. Three hundred and seventy-five microliters of the aqueous phase was collected, placed in new tubes, and evaporated at 43°C in a SpeedVac (Savant Instruments, Holbrook, NY). The dried residue was reconstituted in 100 μ l of 0.005 M H₃PO₄ and centrifuged at 9000g for 1 min. The clear extract was placed in autosampler vials in a refrigerated autosampler (SIL-10A; Shimadzu Scientific Instrument, Wood Dale, IL) for injection of 50 μ l into the HPLC. Preliminary studies using spiked control plasma demonstrated that the recovery of fluvoxamine, fluoxetine, and norfluoxetine were similar and averaged 66 \pm 2%.

Frontal Cortex Tissue Preparation. Fluoxetine and norfluoxetine levels were measured in frontal cortex tissue from animals that received a saline challenge before sacrifice. Extraction of fluoxetine

and norfluoxetine from rat cortex was carried out at 4°C. Ten microliters of 100 μ M fluvoxamine in 0.01 M H₃PO₄ (internal standard) was added to the tissue, which was then sonicated for 5 s (25 W output with microtip) in approximately 10 volumes of ice-cold acetone/1 M formic acid (85:15). After centrifugation at 15,000g for 5 min, the supernatant was collected and evaporated to approximately one-third of the original volume in a SpeedVac (Savant Instruments). An additional 50 μ l of H₂O was added and then centrifuged at 15,000g for 5 min. The clear supernatant was placed in autosampler vials in a refrigerated autosampler (SIL-10A; Shimadzu Scientific Instruments) for injection of 35 μ l into the HPLC. Preliminary studies using spiked control tissue demonstrated that the recovery of fluvoxamine, fluoxetine, and norfluoxetine were similar and averaged 68 \pm 1%.

HPLC Conditions. Fluoxetine and norfluoxetine were separated by reverse-phase HPLC. The mobile phase consisted of 0.02 M NaH₂PO₄, 0.02 M sodium citrate, 200 mM EDTA, 35% acetonitrile, and 4.4 mM tetraethylammonium (hydrogen sulfate salt) and was brought to an apparent pH of 4.5 using H₃PO₄. The stationary phase consisted of an Ultrasphere ODS 3- μ m reverse-phase 4.6 cm ID \times 7.5 mm L column (Beckman Instruments, Fullerton, CA). Flow rate was adjusted to 0.9 ml/min. Under these conditions, the retention times of fluvoxamine, norfluoxetine, and fluoxetine were 3.0, 3.8, and 4.5 min, respectively. Compounds were detected by ultraviolet absorbance at 230 nm using a UV detector (SPD-10A; Shimadzu Scientific Instruments). Quantitation was achieved by comparing peak heights of samples with peak heights of standards. The on-column limits of detection for norfluoxetine and fluoxetine were 1 and 1.5 pmol, respectively (3:1 signal/noise ratio). The limit of quantitation in plasma was 10 nM for fluoxetine and 6 nM for norfluoxetine. The limit of quantitation in brain tissue was 0.15 pmol/mg wet wt. for fluoxetine and 0.2 pmol/mg wet wt. for norfluoxetine.

Statistical Analyses

Hormone data were analyzed by two-way analyses of variance (ANOVA). G protein levels were analyzed by one-way ANOVA. Group means were compared by Newman-Keuls' multiple range test (Steel and Torrie, 1960). GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD) was used for all statistical analyses.

Results

Hormone Responses to 8-OH-DPAT

Basal levels of oxytocin (Fig. 1A) and ACTH (Fig. 1B) were unchanged through 60 days of withdrawal from fluoxetine. In animals that received no fluoxetine, 8-OH-DPAT produced a significant 15-fold increase in the plasma levels of oxytocin (Fig. 1A) and ACTH (Fig. 1B). Two days after discontinuation of fluoxetine injections, the oxytocin and ACTH responses to 8-OH-DPAT were significantly inhibited (by 74% and 68%, respectively). The magnitude of the inhibited oxytocin response to 8-OH-DPAT gradually increased toward control levels throughout the 60-day withdrawal period, but never reached control levels. At 60 days of withdrawal from fluoxetine, the oxytocin response to 8-OH-DPAT was still significantly lower (by 26.1%) than the oxytocin response in rats that never received fluoxetine. The ACTH response to 8-OH-DPAT was significantly inhibited from 2 through 7 days of fluoxetine withdrawal. By 14 days after the last fluoxetine injection, the ACTH response to 8-OH-DPAT (84% of controls) was not significantly different from the ACTH response in control rats. In addition, although the hormone responses to 8-OH-DPAT at 2 days after discontinuation of fluoxetine were significantly inhibited, the responses were not completely blocked.

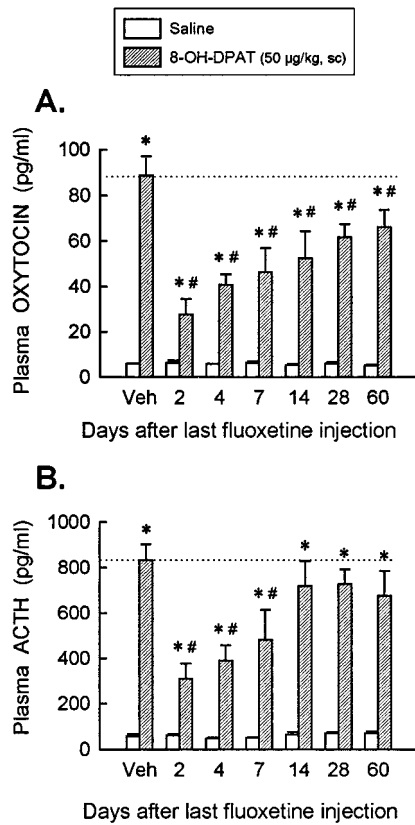


Fig. 1. Oxytocin (A) and ACTH (B) responses to 8-OH-DPAT during withdrawal from fluoxetine. The data represent the mean \pm S.E.M. of six to eight rats per group. The two-way ANOVA for oxytocin indicated a significant effect of withdrawal ($F(6,89) = 5.71, p < .01$), a significant effect of 8-OH-DPAT ($F(1,89) = 249.47, p < .01$), and a significant withdrawal \times 8-OH-DPAT interaction ($F(6,89) = 5.89, p < .01$). The two-way ANOVA for ACTH indicated a significant effect of withdrawal ($F(6,86) = 5.03, p < .01$), a significant effect of 8-OH-DPAT ($F(1,86) = 244.11, p < .01$), and a significant withdrawal \times 8-OH-DPAT interaction ($F(6,86) = 4.58, p < .01$). The Newman-Keuls' post hoc analyses demonstrated: *, significant difference from rats challenged with saline (0 dose of 8-OH-DPAT), $p < .01$; #, significant difference from vehicle rats (0 dose of fluoxetine) challenged with 8-OH-DPAT, $p < .01$.

G Proteins in the Hypothalamus

Examples of immunoblots for G_{i1} and G_{i2} proteins in the hypothalamus are presented in Fig. 2. At 2 days of withdrawal from fluoxetine, mean values (presented in Fig. 3) for the levels of three of the G proteins measured were observed to be lower than control levels; G_z protein levels were 89% of control, G_{i1} protein levels were 90% of control, and G_{i3} proteins were 91% of control. However, statistical analyses indicated that hypothalamic levels of G_z (Fig. 3A), G_{i1} (Fig. 3B), G_{i2} (Fig. 3C), and G_{i3} (Fig. 3D) proteins were not significantly

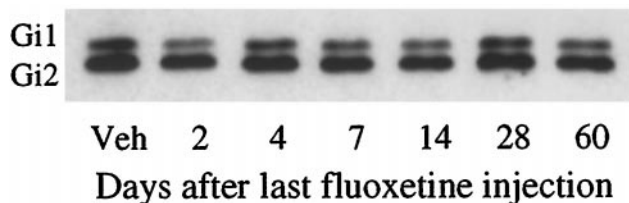


Fig. 2. Example of immunoblot of G_{i1} and G_{i2} proteins in the hypothalamus of rats at various times after discontinuation of fluoxetine injections.

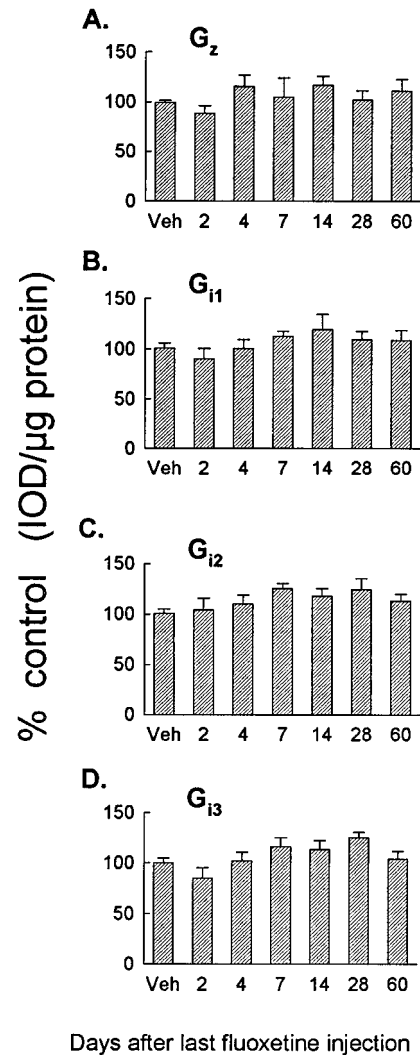


Fig. 3. G proteins in the hypothalamus during withdrawal from fluoxetine. The data represent the mean \pm S.E.M. of seven to eight rats per group. The one-way ANOVA indicated no significant effect of withdrawal for G_z ($F(6,46) = 0.73, p > .10$), G_{i1} ($F(6,42) = 1.02, p > .10$), G_{i2} ($F(6,47) = 1.33, p > .10$), or for G_{i3} ($F(6,44) = 2.14, p > .05$).

different from controls at any time point during withdrawal from fluoxetine.

Fluoxetine and Norfluoxetine in Plasma and Brain Tissue

Fluoxetine and norfluoxetine concentrations in the plasma and in brain tissue are presented in Table 1. Fluoxetine concentrations were below the detection limit (10 nM) in plasma even at 2 days of withdrawal from fluoxetine injections. Norfluoxetine concentrations (detection limit 6 nM) were high (439.53 ± 99.68 nM) 2 days after the last fluoxetine injection. At 4 days, low levels of norfluoxetine were detected in two out of the seven animals, and levels were undetectable in the remaining five rats (mean of two rats with detectable levels was 13.0 ± 3.0 nM). Norfluoxetine concentrations were below the detection limit in plasma from 7 to 60 days of withdrawal from fluoxetine.

In brain tissue, low concentrations of fluoxetine were detected in four of seven animals 2 days after the last fluoxetine injection; levels in the remaining three rats were undetectable (mean of four rats with detectable levels was 0.23 ± 0.06

TABLE 1
Plasma and brain tissue concentrations of fluoxetine and norfluoxetine

No. of Days after Last Fluoxetine Injection	Plasma ^a		Brain tissue ^b	
	FLU	NOR	FLU	NOR
	nM		pmol/mg	
Vehicle	nd	nd	nd	nd
2	nd	439.53 ± 99.68	0.13 ± 0.06 ^c	41.42 ± 9.43
4	nd	3.71 ± 2.49 ^d	nd	0.36 ± 0.08
7	nd	nd	nd	0.09 ± 0.05 ^e
14	nd	nd	nd	nd
28	nd	nd	nd	nd
60	nd	nd	nd	nd

FLU, fluoxetine; NOR, norfluoxetine; nd, not detectable. Data represent mean ± S.E.M. of seven to eight rats per group.

^a Detection limit in plasma was 10 nM for fluoxetine, 6 nM for norfluoxetine.

^b Detection limit in brain tissue was 0.15 pmol/mg wet weight tissue for fluoxetine, 0.20 pmol/mg for norfluoxetine.

^c Mean represents two of seven rats with levels near the detection limit (mean = 13.0 ± 3.0 nM) and the remaining five rats with undetectable levels.

^d Mean represents four of seven rats with levels near the detection limit (mean = 0.23 ± 0.06 pmol/mg) and the remaining three rats with undetectable levels.

^e Mean represents three of eight rats with levels near the detection limit (mean = 0.24 ± 0.03 pmol/mg) and the remaining five rats with undetectable levels.

pmol/mg wet wt. tissue). Tissue concentrations of fluoxetine were below the detection limit (0.15 pmol/mg) through the remaining 60 days of withdrawal from fluoxetine. Norfluoxetine concentrations (detection limit 0.20 pmol/mg) were high (41.42 ± 9.43 pmol/mg) 2 days after discontinuation of fluoxetine injections. Low concentrations (0.36 ± 0.08 pmol/mg wet weight tissue) were detected at 4 days after the last fluoxetine injection, and mean concentrations were at or below the detection limit through the remaining 60 days of withdrawal from fluoxetine.

Discussion

Consistent with our previous results (Li et al., 1993b, 1996), long-term exposure to fluoxetine induces an inhibition in the hormonal responses to the 5-HT_{1A} agonist 8-OH-DPAT. Results from the present study indicate that the inhibition of the oxytocin response to 8-OH-DPAT persists through 60 days of withdrawal from fluoxetine, and inhibition of the less-sensitive ACTH response persists through at least 7 days of withdrawal. In addition, this sustained inhibition occurs in the absence of fluoxetine or norfluoxetine in the blood and in brain tissue. Furthermore, desensitization during the withdrawal period was not accompanied by reductions in G_z or G_i protein levels in the hypothalamus as was observed during exposure to fluoxetine in our previous studies (Li et al., 1996, 1997; Raap et al., 1998). These observations suggest that changes in levels of G_z or G_i proteins are not the sole underlying mechanism of 5-HT_{1A} receptor system desensitization during fluoxetine withdrawal.

Discontinuation of SSRIs in clinical practice has received little attention. A concern in the management of SSRI discontinuation is the lack of predictability for the need to resume medication if the original symptoms do recur. Although the somatic and psychological symptoms during discontinuation are becoming characterized, physiological indicators such as neuroendocrine challenge tests might provide a means for monitoring underlying changes in neuronal function during withdrawal.

Neuroendocrine challenge tests can provide an indicator of receptor system function in the brain. Oxytocin and ACTH, which are controlled by receptor systems in the hypothalamus,

are released into the circulation in response to a challenge with 5-HT_{1A} agonists such as 8-OH-DPAT, buspirone, ipsapirone, and alnespirone (Levy et al., 1995; Bagdy, 1996; Van de Kar et al., 1998a; Vicentic et al., 1998). In addition, the 5-HT_{1A} agonist-induced increases in levels of these hormones represent an amplification of receptor function in the hypothalamus; hence, small alterations in receptor function are easily detected. Measuring plasma levels of these hormones in response to 5-HT_{1A} agonists has contributed significantly to understanding the desensitization of hypothalamic postsynaptic 5-HT_{1A} receptor systems after long-term treatment with SSRIs in humans (Lesch et al., 1991; Sargent et al., 1997) and in rats (Cole et al., 1990; Li et al., 1993b, 1996, 1997). In the present study, the sustained inhibition of the hormone responses to 8-OH-DPAT indicates a lasting desensitization of postsynaptic 5-HT_{1A} receptors in the hypothalamus during withdrawal from long-term fluoxetine treatment in rats. It is possible that neuroendocrine challenge tests can be used in humans during administration of SSRIs as well as after discontinuation to monitor receptor system function in the brain.

Although changes in the sensitivity of 5-HT_{1A} receptor systems may be more accurately determined from a dose-response curve of 8-OH-DPAT, the single dose of 8-OH-DPAT selected in the present experiments is very reliable as an indicator of 5-HT_{1A} receptor sensitivity. Maximal stimulation of the secretion of oxytocin and ACTH is reached by 8-OH-DPAT doses of 200 to 500 µg/kg (s.c.) (Li et al., 1993b). A submaximal dose of 8-OH-DPAT was used in the present study (50 µg/kg, s.c.) to prevent activation of other 5-HT receptor subtypes. This dose of 8-OH-DPAT was previously shown to be the most effective dose in demonstrating desensitization of 5-HT_{1A} receptors (Li et al., 1996).

The difference between the time course of the oxytocin and ACTH responses to 8-OH-DPAT during fluoxetine withdrawal is likely due to differences in the amplification and hence, the sensitivity of the response to activation of 5-HT_{1A} receptors. The oxytocin neurons in the hypothalamus send projections into the posterior pituitary, releasing oxytocin directly into the bloodstream upon 5-HT_{1A} receptor activation (Bagdy, 1996). In contrast, cells that release ACTH are located in the anterior lobe of the pituitary gland. Release of ACTH into the bloodstream is dependent upon activation of corticotrophin-releasing hormone (CRH) receptors on pituitary corticotrophs (Turnbull and Rivier, 1997). CRH reaches the pituitary via pituitary portal vessels upon release from neurons in the hypothalamus, which respond to 8-OH-DPAT (Calogero et al., 1989). Hence, the release of ACTH in response to 8-OH-DPAT is more indirect, and thereby more amplified, than the release of oxytocin. Therefore, small reductions in 8-OH-DPAT-induced CRH release after long-term fluoxetine treatment might not be as detectable as small reductions in oxytocin release. As the desensitization of 5-HT_{1A} receptors gradually dissipates after discontinuation of fluoxetine, it would be expected that plasma levels of a more indirect, amplified peripheral marker of 5-HT_{1A} receptor function would return to control levels more quickly. The more rapid return of the ACTH response to 8-OH-DPAT as compared with the oxytocin response observed in the present study supports this conclusion. These results further suggest that measuring oxytocin responses to 5-HT_{1A} agonists in humans may provide a more sensitive indicator of the func-

tion of 5-HT_{1A} receptor systems in the hypothalamus than measuring plasma levels of ACTH. The reduced neuroendocrine responses to 5-HT_{1A} agonists are not due to a general reduction in neuroendocrine systems; this treatment with fluoxetine produces an increase in the neuroendocrine responses to the 5-HT_{2A/2C} agonist (\pm)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (Li et al., 1993a).

The desensitization of the hormone responses to 8-OH-DPAT persisted in the *absence* of detectable levels of fluoxetine and its active metabolite norfluoxetine in plasma and in brain tissue. The observed levels of fluoxetine and norfluoxetine and their elimination from brain tissue after discontinuation of fluoxetine are similar to those reported by Gardier et al. (1994) after a 21-day, 10-mg/kg fluoxetine treatment regimen in rats. Other studies have reported various pharmacokinetic, behavioral, and biochemical changes that persist after discontinuation of long-term fluoxetine (Wong et al., 1975; Stolz et al., 1983; Caccia et al., 1992, 1997; Trouvin et al., 1993); treatment regimens and time course of study after discontinuation, however, differed from the present parameters and made comparisons difficult. In the present study, the ACTH response was still inhibited 7 days after the last fluoxetine injection, at a time when there were virtually no detectable levels of norfluoxetine in brain tissue, and at least 3 days after the levels of norfluoxetine in the plasma were undetectable. In addition, the oxytocin response was still reduced 60 days after discontinuation of fluoxetine injections, long after the disappearance of fluoxetine and its metabolite. Hence, the sustained desensitization is not dependent upon the continued presence of detectable levels of fluoxetine or norfluoxetine in the blood or in brain tissue.

The 5-HT_{1A} receptors are coupled to the G_{v/o/z} family of proteins, which link the receptor to effector mechanisms (Raymond et al., 1993; Barr et al., 1997). Our studies using pertussis toxin and G_z antisense oligodeoxynucleotides indicate that hypothalamic G_z proteins mediate the 5-HT_{1A} receptor-induced increase in ACTH and oxytocin secretion (Serres et al., 1998; Van de Kar et al., 1998b). We have previously reported a dose-dependent decline in the hypothalamic levels of G_z proteins measured 18 h after 14 days of fluoxetine injections (Raap et al., 1999). Therefore, G_z proteins might play a role in fluoxetine-induced desensitization of hypothalamic postsynaptic 5-HT_{1A} receptors. However, the sustained inhibition of the neuroendocrine responses to a 5-HT_{1A} agonist are not paralleled by a reduction in G_z protein levels in the hypothalamus during withdrawal from long-term fluoxetine. In the current study, there were no reductions in the levels of G_z proteins even 48 h after fluoxetine injections stopped, at a time when desensitization of postsynaptic 5-HT_{1A} receptors was observed. Taken together with results from our previous studies, these findings suggest that the adaptive changes contributing to the gradual development of postsynaptic 5-HT_{1A} receptor desensitization during SSRI-induced blockade of 5-HT uptake may differ from the mechanisms responsible for the maintenance of reduced 5-HT_{1A} receptor function during withdrawal. One possibility is that the reduction of G proteins in the hypothalamus can only be maintained when sufficient levels of fluoxetine, or its bioactive metabolite, norfluoxetine are present to maintain inhibition of 5-HT reuptake. In previous studies, we observed that hypothalamic levels of G_z and G_{i1} proteins were significantly reduced 18 h after the last fluox-

etine injection (Raap et al., 1998). The plasma levels of norfluoxetine, measured 2 days after the last injection of fluoxetine are less than 50% of those observed 18 h after the last fluoxetine injection (Raap et al., 1999). It is further possible that the desensitization during fluoxetine exposure and the sustained desensitization after cessation of fluoxetine treatment are induced by a common unknown mechanism.

Although reductions in G_z protein levels may contribute to the desensitization of 5-HT_{1A} receptors, the mechanisms involving the *sustained* desensitization through 60 days of withdrawal appear to involve more than changes in *levels* of G proteins. It is possible that reduced G_z and G_i protein levels during fluoxetine exposure initiate a cascade of events in the postsynaptic neuron in the hypothalamus that maintain the desensitized postsynaptic 5-HT_{1A} receptors during withdrawal. Such mechanisms may include post-translational modifications of G proteins, resulting in alterations in the regulation of G protein coupling to 5-HT_{1A} receptors, or in the reduced ability of G proteins to couple with effector mechanisms. Phosphorylation of serine residues of G_z proteins at positions 16 and 27 has been described *in vitro* (Fields and Casey, 1995; Ho and Wong, 1997) and could be responsible for reduced coupling of G_z proteins to 5-HT_{1A} receptors or to effector enzymes. Additionally, changes in palmitoylation or myristoylation of G_z proteins may also contribute to reduced coupling of G_z proteins to 5-HT_{1A} receptors (Hallak et al., 1994; Tu et al., 1997).

In summary, the maintenance of the desensitization of postsynaptic 5-HT_{1A} receptors after discontinuation of fluoxetine likely involves mechanisms different from those contributing to the development of the desensitization during exposure. Furthermore, current results provide support for the use of neuroendocrine challenge tests in humans as a useful means of measuring the function of postsynaptic 5-HT_{1A} receptors in the hypothalamus both during and after exposure to SSRIs. The desensitization of hypothalamic postsynaptic 5-HT_{1A} receptors may be important for the therapeutic effectiveness of SSRIs. Thus, comparing symptoms of withdrawal with the results from neuroendocrine challenge tests in humans could provide a diagnostic tool for monitoring neuronal function after discontinuation of SSRIs and predicting a need for readministration of SSRIs.

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