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Pharmacology

Blockage of $5HT_{2C}$ serotonin receptors by fluoxetine (Prozac)

(membrane currents/receptor binding/*Xenopus* oocytes/HeLa cells)

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ABSTRACT **Fluoxetine (Prozac) inhibited the membrane currents elicited by serotonin (5-hydroxytryptamine; 5HT) in *Xenopus* oocytes expressing either cloned $5HT_{2C}$ receptors or 5HT receptors encoded by rat cortex mRNA. Responses of $5HT_{2C}$ receptors, elicited by nM concentrations of 5HT, were rapidly and reversibly blocked by micromolar concentrations of fluoxetine. For responses elicited by 1 μM 5HT, the IC_{50} of fluoxetine inhibition was $\approx$20 μM. In accord with the electrophysiological results, fluoxetine inhibited the binding of [^{3}H]5HT to $5HT_{2C}$ receptors expressed in HeLa cells ($K_i \approx$ 65–97 nM), and the binding to 5HT receptors in rat cortex membranes was also inhibited but less efficiently ($K_i \approx$ 56 μM). Our results show that fluoxetine is a competitive and reversible antagonist of $5HT_{2C}$ receptors and suggest that some therapeutic effects of fluoxetine may involve blockage of 5HT receptors, in addition to its known blockage of 5HT transporters. Similar work may help to design more selective compounds for use in the treatment of brain disorders.**

Fluoxetine (Prozac) is widely used in the treatment of a variety of brain disorders, such as mental depression, panic disorder, obesity, and alcoholism. It is generally believed that fluoxetine exerts its therapeutic effects by enhancing serotonergic transmission, exclusively through inhibition of serotonin (5-hydroxytryptamine; 5HT) transporters with minimal or no effects on other neurotransmitter receptors (1, 2). However, it has been shown that fluoxetine inhibits 5HT binding in the choroid plexus (3), that it appears to be an agonist of $5HT_{2C}$ receptors in cultured astrocytes (4), and that it inhibits currents mediated by $5HT_3$ receptors in rat nodose ganglion neurons (5), as well as the binding of 5HT to $5HT_3$ and $5HT_4$ receptors (6). In addition, chronic treatment of fluoxetine may cause a down-regulation of $5HT_1$ receptors (2) and also alter the expression of other receptors, although the latter effects are somewhat controversial (7).

$5HT_{2C}$ receptors [formerly termed $5HT_{1C}$, (8)] are widely expressed in the brain and spinal cord, are particularly enriched in the choroid plexus (9), and appear to mediate many important effects of 5HT. For example, transgenic mice that are devoid of $5HT_{2C}$ receptors are overweight and are prone to seizure-induced death, suggesting a role for this type of receptor in the control of appetite and neuronal network excitability (10). Given fluoxetine's multiple therapeutic effects on a variety of mental and eating disorders, it was important to study in more detail the action of fluoxetine on $5HT_{2C}$ receptors.

MATERIALS AND METHODS

RNA *in Vitro* Transcription. *Not*I-linearized pSR1c (11) was transcribed using T7 RNA polymerase (Promega) in the presence of a cAMP-binding protein analog $m^7G(5')ppp(5')G$ (Pharmacia). Rat cortex RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (12), and poly(A)$^+$ RNA was obtained by oligo(dT)-cellulose chromatography.

Translation in *Xenopus* Oocytes and Electrophysiological Recording. Oocytes were injected with mRNA, and recordings were made 4–10 days later, essentially as described (13–15). Briefly, oocytes were injected with 1 ng of cloned rat $5HT_{2C}$ mRNA, or with 50 ng of rat cortex mRNA, and kept in Barth's medium containing 0.01 mg/ml gentamicin. Two days later, the oocytes were treated with collagenase to remove the follicular and other enveloping cells (13, 15). During the subsequent days, membrane currents were recorded, usually with the membrane potential clamped at -60 mV, digitized, and stored for analyses. Drugs were applied via continuous bath superfusion of Ringer's solution at 5–8 ml/min (bath volume $\approx$ 100 μl).

Transfection and Membrane Preparation. The *Eco*RI fragment of pSR1c, containing the entire rat $5HT_{2C}$ receptor coding region, was subcloned into a eukaryotic expression vector pcDNA3 (Invitrogen), and transfected into HeLa cells by electroporation (Bio-Rad Gene Pulser, 500 μF, 300 V). Cell membranes were prepared according to Albert *et al.* (16) with slight modifications. Briefly, 2 days after transfection, the cells were harvested in a hypotonic buffer (50 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$) and precipitated at 25,000 $\times$ *g* for 30 min at 4°C. Membrane pellets were washed once, resuspended in a receptor binding assay buffer (50 mM Tris·HCl, pH 7.4/4 mM $CaCl_2$/0.1% ascorbic acid), and stored at -70°C until use. Membrane protein was measured as described by Bradford (17).

Rat cerebral cortex membranes were prepared by homogenizing dissected cortex with a loose fitting Polytron in 0.32 M sucrose, precipitating at 800 $\times$ *g* for 10 min, and then reprecipitating the supernatant at 25,000 $\times$ *g* for 30 min. The membrane pellet was washed twice with 1 mM EGTA and resuspended in the receptor binding buffer.

Receptor Binding Assay. Cell membranes (25–50 μg protein) were incubated with [^{3}H]5HT ($\approx$75 Ci/mmol; 1 Ci = 37 GBq; Amersham) in the binding assay buffer (200 μl) at 4°C for 30 min. Nonspecific binding was determined by adding 100 μM 5HT together with [^{3}H]5HT. Binding assays were terminated by centrifugation, and the membranes were solubilized with a tissue solubilizer (TS-2, Research Product International) before quantification by liquid scintillation spectrometry ($\approx$30% efficiency). In competition assays, different concentrations of fluoxetine were used in the reaction. K_i was calculated from the equation: $K_i = IC_{50}/(1 +$ radioligand concentration$/K_d$ of the radioligand) (18), where IC_{50} is the concentration of the competing ligands required for 50% inhibition of the radioligand binding. Data were analyzed using the program PRISM (GraphPad Software, San Diego).

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Abbreviation: 5HT, 5-hydroxytryptamine (serotonin).

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RESULTS

Effects of Fluoxetine on $5HT_{2C}$ Receptor-Mediated Responses. Defolliculated oocytes from *Xenopus* frogs do not have native 5HT receptors, although, very rarely, 5HT elicits small oscillatory currents in some oocytes (R.M., unpublished results). In the experiments reported here, all the control, noninjected oocytes from more than 20 donor frogs did not respond to 5HT. In contrast, oocytes injected with $5HT_{2C}$ mRNA gave large membrane currents in response to 5HT. Fluoxetine applied alone at concentrations up to 100 μM did not induce a membrane current response or, occasionally, it elicited a very small inward current (Fig. 1). When fluoxetine was applied together with 5HT, the responses to 10 nM 5HT were completely abolished and those to 10 μM 5HT, a concentration that elicits near maximal responses in *Xenopus* oocytes, were greatly attenuated (Figs. 1 and 2*A*). The blocking effect of fluoxetine was rapid in onset and was also rapidly reversible. For example, in Fig. 1, the response to 10 μM 5HT had already recovered to near its control level 17 min after removal of fluoxetine (Fig. 1, lower trace). However, in some oocytes, the responses to 5HT did not recover fully even 1 h after exposure to high concentrations of fluoxetine. A dose-response analysis of fluoxetine inhibition of the membrane current responses elicited by 1 μM 5HT gave an IC_{50} of $\approx$20 μM (Fig. 3).

It is known that injection of rat cerebral cortex mRNA into oocytes leads to the expression of functional 5HT receptors (19), whose molecular types have not yet been clearly established, although the $5HT_{2C}$ receptor seems to be the predominant 5HT receptor subtype in the rat central nervous system (20). Therefore, we decided to see if the 5HT receptors expressed by rat cortex mRNA were also blocked by fluoxetine. Here again, fluoxetine (100 μM) blocked completely the responses to 10 nM 5HT and greatly reduced the responses to 10 μM 5HT (Fig. 2*B*).

Fluoxetine was an effective blocker even at relatively low concentrations. For example, in the oocyte used for Fig. 4, 1 μM fluoxetine exerted a rapid and fully reversible inhibition of the response to 2 nM 5HT. On average, the amplitude of the response to 2 nM 5HT was reduced $\approx$50% by a coapplication with 1 μM fluoxetine, and a second coapplication of fluoxetine exerted a similar inhibitory effect. Moreover, when 1 μM or 10 μM fluoxetine was applied briefly during prolonged application of low concentrations of 5HT, the responses were blocked rapidly and almost completely (Fig. 5 *A* and *C*), and the 5HT current recovered rapidly after removing the fluoxetine (Fig. 5*A*).

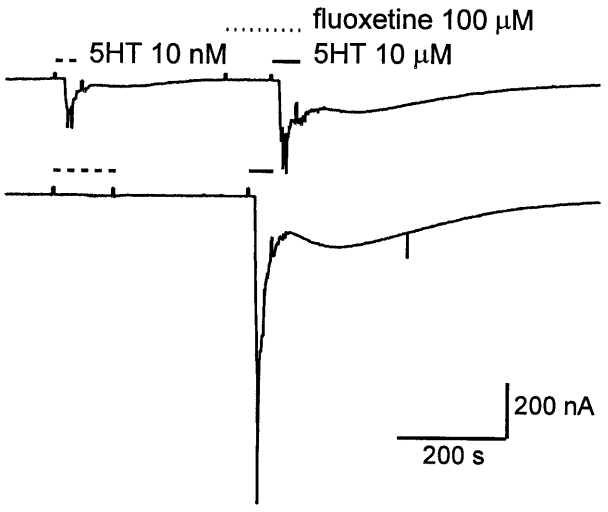

FIG. 1. Inhibition of $5HT_{2C}$ currents by fluoxetine (100 μM) in an oocyte expressing cloned $5HT_{2C}$ receptors (upper trace). The continuing lower trace shows that the response to 10 nM 5HT was still inhibited while that to 10 μM 5HT had recovered substantially after the removal of fluoxetine. For this and following figures, the oocyte's membrane potential was held at −60 mV. Inward currents are represented by downward deflections, and drug applications are indicated by bars above the traces.

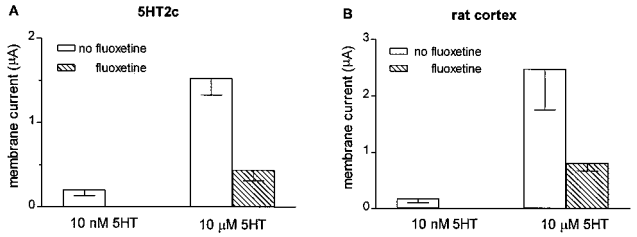

FIG. 2. Blocking effects of fluoxetine (100 μM) on 5HT currents in oocytes injected with cloned $5HT_{2C}$ receptor mRNA (*A*) or rat cortex mRNA (*B*). For these experiments, fluoxetine and 5HT were coapplied for 1–2 min. Results are mean ± SE from 3–10 oocytes. In both *A* and *B*, there were no detectable responses to coapplications of 10 nM 5HT and fluoxetine.

Effects of Fluoxetine on Other Receptors Also Linked to the Phosphoinositide Pathway. The membrane current responses to 5HT in *Xenopus* oocytes, injected with either rat cortex mRNA or cloned $5HT_{2C}$ RNA, result from activation of an endogenous receptor-channel coupling pathway. The binding of 5HT to $5HT_{2C}$ receptors activates the phosphoinositide pathway via a G protein, thus leading to the formation of inositol triphosphate and the release of Ca^{2+} from intracellular stores (21). This Ca^{2+} in turn opens Ca^{2+}-gated Cl^- channels (14, 19, 22, 23). Therefore, it was possible that fluoxetine inhibited the membrane currents elicited by 5HT by acting at

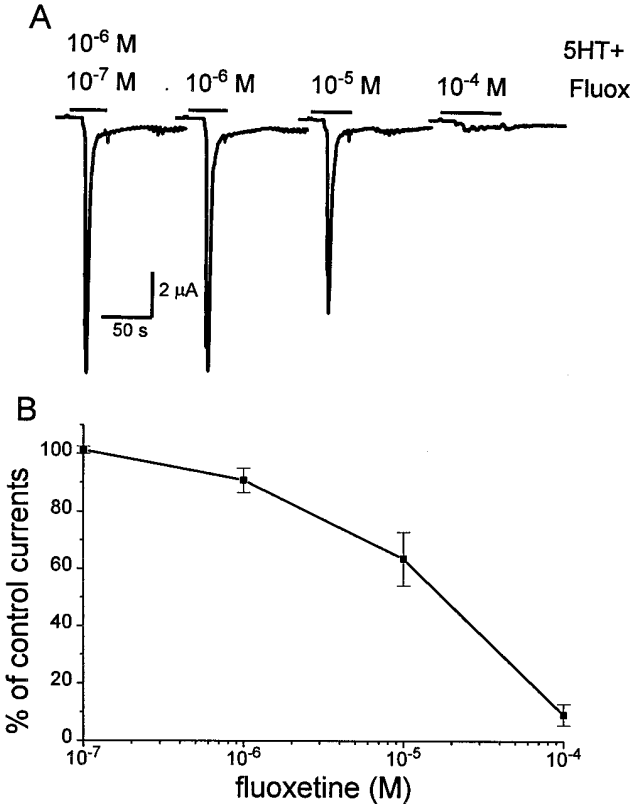

FIG. 3. Dose-response relation of fluoxetine inhibition of responses to 5HT in oocytes expressing $5HT_{2C}$ receptors. (*A*) Sample traces from one oocyte. (*B*) Inhibition by different concentrations of fluoxetine coapplied with 1 μM 5HT. Each point represents mean ± SE from four to seven oocytes from one donor frog.

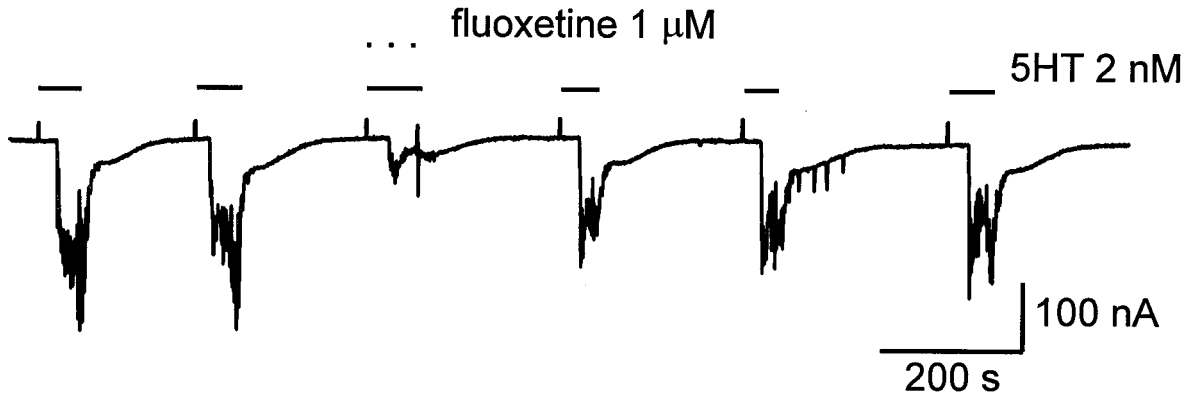

FIG. 4. Inhibitory effect of 1 μM fluoxetine on $5HT_{2C}$ currents. 5HT was usually applied several times until a stable response was reached before coapplying fluoxetine.

any of the multiple steps on the receptor-channel coupling pathway. To examine this possibility we used two approaches. In one, we studied the effects of fluoxetine on the responses mediated by native or expressed angiotensin II receptors or by native serum factor receptors, all of which activate the same phosphatidylinositol pathway used by the $5HT_{2C}$ receptors (24, 25). In the other, we studied the effect of fluoxetine on the binding of 5HT to the $5HT_{2C}$ receptors.

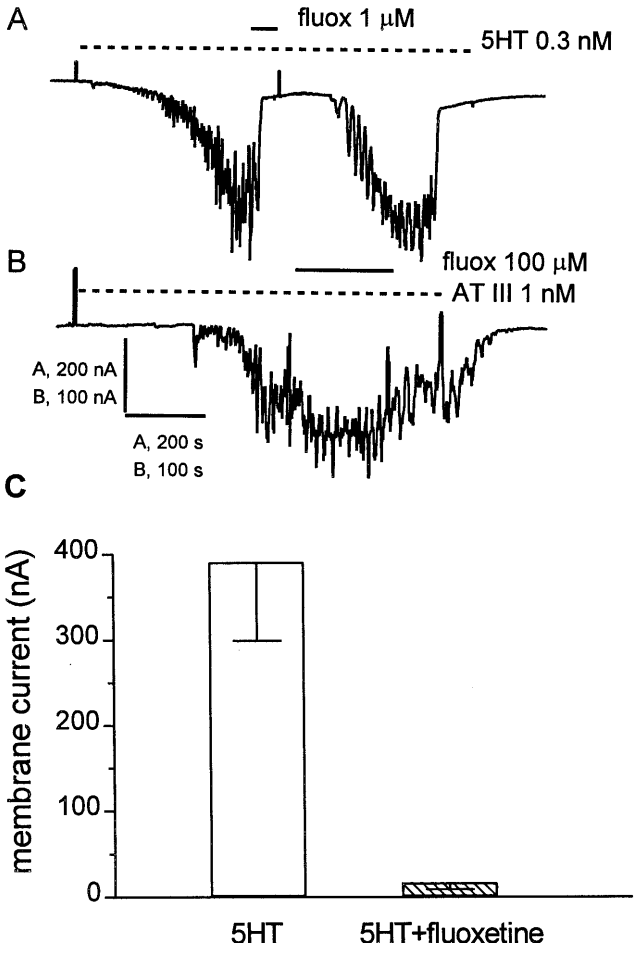

FIG. 5. Inhibition of $5HT_{2C}$ currents by a low concentration of fluoxetine (1 μM). (*A*) Sample trace of a nearly complete inhibition of $5HT_{2C}$ current. (*B*) Sample trace of a high concentration of fluoxetine (100 μM) not inhibiting the current elicited by angiotensin III (1 nM) activation of angiotensin II receptors. In this and the following figure, angiotensin II receptors were expressed by injecting 5 ng of bovine AT1 receptor mRNA. (*C*) Fluoxetine (10 μM) blockage of $5HT_{2C}$ currents elicited by 0.3 nM 5HT. Results are mean ± SE from six to seven oocytes.

As illustrated in Fig. 5, the responses to 0.3 nM 5HT were almost completely blocked by a 1 μM concentration of fluoxetine (Figs. 5 *A* and *C*). In contrast, the response to 1 nM angiotensin III was not inhibited, and may even be increased slightly, by a 10^5-fold higher concentration of fluoxetine (100 μM) (Fig. 5*B*). Similarly, the oscillatory current responses elicited by serum were not blocked by 100 μM fluoxetine (Fig. 6). All this suggested strongly that fluoxetine did not affect appreciably the phosphatidylinositol receptor-channel coupling pathway. Therefore, it appeared very likely that the inhibiting action of fluoxetine on the responses to serotonin was exerted at the receptor level.

Effects of Fluoxetine on [^{3}H]5HT Binding to $5HT_{2C}$ Receptors. To determine whether fluoxetine affects directly the binding of 5HT to its receptors, we examined the effects of fluoxetine on the binding of [^{3}H]5HT to membranes from HeLa cells transiently expressing $5HT_{2C}$ receptors and to membranes from rat cerebral cortex. To reduce complications from potential binding to 5HT transporters, all these experiments were carried out at 4°C and in a Na^+-free binding assay buffer. Under these conditions, the binding of fluoxetine to 5HT transporters should be less than 0.5% of that occurring under more physiological conditions (37°C and with Na^+ present) (26). Fluoxetine inhibited the binding of [^{3}H]5HT to $5HT_{2C}$ receptors with a relatively high efficiency. Thus, when 3.2 nM [^{3}H]5HT was used to label the $5HT_{2C}$ receptors, fluoxetine inhibited the binding at concentrations as low as 10 nM, with an IC_{50} of 0.11 ± 0.01 μM ($K_i \approx 65$ nM, $n = 3$). At close to saturating concentrations for binding of [^{3}H]5HT (18 nM), the IC_{50} was 0.51 ± 0.06 μM ($K_i \approx 97$ nM, $n = 3$). At both concentrations of [^{3}H]5HT, the specific binding was completely inhibited by 100 μM fluoxetine (Fig. 7). In membranes from HeLa cells expressing $5HT_{2C}$ receptors, the K_d of

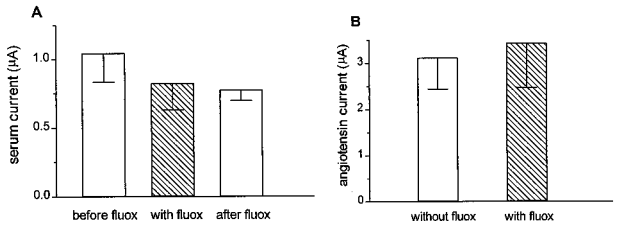

FIG. 6. Effect of fluoxetine on other native or expressed receptors that also utilize the phosphatidylinositol pathway. (*A*) Effect of fluoxetine on the response elicited by rabbit serum (1/1000 dilution) in noninjected oocytes. (*B*) Effect of fluoxetine on cloned bovine angiotensin II receptor-mediated responses. Angiotensin III (1 μM) was applied alone or together with fluoxetine (100 μM). Results are mean ± SE from three to four oocytes.

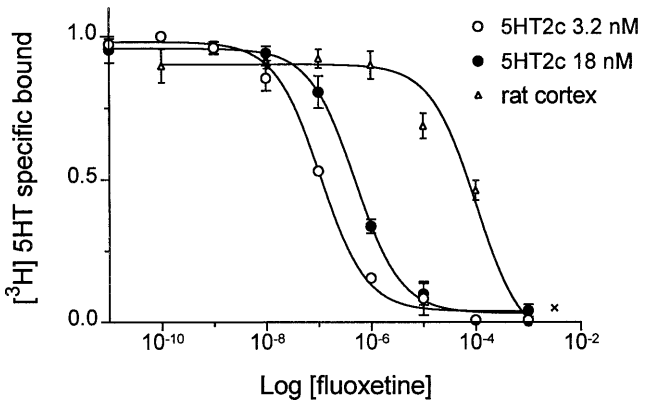

FIG. 7. Competition of fluoxetine with [^{3}H]5HT for binding to $5HT_{2C}$ receptors expressed in HeLa cells or to native rat cortex 5HT receptors. As indicated, 3.2 or 18 nM of [^{3}H]5HT was used to label the $5HT_{2C}$ receptors expressed in HeLa cells (circles), and 3.2 nM [^{3}H]5HT was used to label the rat cortex 5HT receptors. The inhibition by 1 mM 5HT (×) is also included to show that the binding of [^{3}H]5HT to $5HT_{2C}$ receptors was completely blocked by fluoxetine. Data points are mean ± SE from three independent experiments in duplicate.

[^{3}H]5HT binding was 4.3 ± 0.8 nM (n = 4), with B_{max} ranging from 1.4 to 6.8 pmol/mg protein. There was no specific [^{3}H]5HT binding in nontransfected HeLa cell membranes.

In agreement with the electrophysiological results, fluoxetine also blocked the binding of [^{3}H]5HT to rat cortical membranes, although with a much lower efficiency as compared with its effect on the $5HT_{2C}$ receptors. As shown in Fig. 7, a higher concentration of fluoxetine (>1 μM) was needed to detect inhibition of [^{3}H]5HT binding to rat cortical membranes and the IC_{50} was 103 ± 18 μM ($K_i \approx 56$ μM, n = 5).

DISCUSSION

This study shows clearly that fluoxetine has a potent blocking effect on $5HT_{2C}$ receptors expressed in both *Xenopus* oocytes and HeLa cells, as well as on the native 5HT receptors present in cerebral cortical cell membranes. The evidence is many fold. (*i*) In *Xenopus* oocytes expressing cloned $5HT_{2C}$ receptors, fluoxetine inhibited rapidly the currents elicited by 5HT. Appreciable inhibition was observed with fluoxetine concentrations lower than 1 μM, and the inhibition was rapidly reversible. For responses induced by 1 μM 5HT, the IC_{50} of fluoxetine inhibition was ≈20 μM. (*ii*) Fluoxetine did not block the oocyte responses to cloned (angiotensin II) or native serum receptors that are mediated by the same receptor-channel coupling pathway used by 5HT. (*iii*) Fluoxetine did not have an agonist action on $5HT_{2C}$ receptors in oocytes because it failed to elicit appreciable currents. (*iv*) In HeLa cell membrane preparations, the binding of [^{3}H]5HT to expressed rat $5HT_{2C}$ receptors was inhibited completely by fluoxetine, with a K_i of ≈65–97 nM. Therefore, in oocytes, as well as in membranes of HeLa cells, fluoxetine acts as a reversible competitive antagonist of recombinant $5HT_{2C}$ receptors.

In addition, our study shows that fluoxetine also blocks the responses of 5HT receptors expressed in oocytes from rat cortex mRNA, and inhibits also the binding of [^{3}H]5HT to 5HT receptors present in rat cortical membranes. So far, about 10 different subtypes of 5HT receptors have been found in rat brain (27), including the $5HT_{2C}$ receptor. Our results show that the K_i of fluoxetine for rat cortex 5HT receptors is very low compared with that for $5HT_{2C}$ receptors (56 μM versus 65 nM). This suggests that fluoxetine has a stronger influence on the $5HT_{2C}$ receptor than on the other subtypes of 5HT receptors present in rat cortex. Furthermore, this disparity in inhibitory potency between recombinant $5HT_{2C}$ receptors and rat cortical 5HT receptors suggests that the main 5HT receptor in the rat cortex may not be of the $5HT_{2C}$ type.

So far, the therapeutic effects of fluoxetine have been attributed primarily to its inhibition of 5HT transporters. Interestingly, it has been shown that the therapeutic plasma concentration of fluoxetine is in the micromolar range (28), and our studies show that, at this concentration range, fluoxetine can potently inhibit the membrane current responses mediated by $5HT_{2C}$ receptors. Moreover, the affinity of fluoxetine for $5HT_{2C}$ receptors (K_i = 65 nM) is close to its affinity for 5HT transporters (K_i = 33 nM) (29), which is also well below the therapeutic plasma concentration of fluoxetine. Thus, some therapeutic effects of fluoxetine may be a consequence of blocking both 5HT transporters and $5HT_{2C}$ receptors. It should be noted that the blockage of 5HT transporters and that of $5HT_{2C}$ receptors would have opposing actions on serotonergic synaptic transmission. Moreover, in addition to its effects at serotonergic synapses fluoxetine may exert important actions via volume transmission (30) at sites far away from the synaptic regions. In these areas, probably containing extra-junctional receptors (31) of many types, the extracellular concentration of 5HT is very likely much lower, and more sustained, than the concentrations reached within the synaptic gap. Thus, the volume transmission effect of fluoxetine on the extra-junctional receptors may resemble its effect on oocytes exposed to low concentrations of 5HT, where 1 μM fluoxetine blocked almost completely the response to 5HT. Because of the highly nonlinear dose/response relationship of $5HT_{2C}$ receptors the blockage of even a small number of receptors in a cell would lead to very profound changes, not only in its responses to 5HT but also in those to other neurotransmitters which act on receptors linked to the same phosphatidylinositol receptor-channel coupling mechanism (32).

Thus, the mechanisms of the medicinal actions of fluoxetine appear to be more complicated than hitherto anticipated. The results presented here may help not only to advance our understanding of the therapeutic mechanisms of fluoxetine and related drugs, but also in the development of new families of drugs that could lead to improved treatments for depression and other dysfunction of the brain.

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