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Electrophysiological effect of fluoxetine on *Xenopus* oocytes heterologously expressing human serotonin transporter[1]

Hong-wei WANG[2], Ci-zhen LI[2], Zhi-fang YANG[2], Yan-qian ZHENG[2], Ying ZHANG[2], Yuan-mou LIU[2,3]

[2]*Department of Physiology, Shanghai Jiaotong University Medical School, Shanghai 200025, China*

Key words

Abstract

Aim: To investigate the electrophysiological effect of fluoxetine on serotonin transporter. **Methods:** A heterologous expression system was used to introduce human serotonin transporter (hSERT) into *Xenopus* oocytes. A 2-electrode voltage clamp technique was used to study the pharmacological properties of fluoxetine. **Results:** hSERT-expressing oocytes were perfused with 10 μmol/L serotonin (5-HT) to induce hSERT-current. The 5-HT-induced hSERT currents were dose-dependently reversed by fluoxetine. The RC_{50} (concentration that achieved a 50% reversal) was approximately 3.12 μmol/L. Fluoxetine took more time to combine with hSERT than 5-HT did, and it was also slow to dissociate from hSERT. This long-lasting effect of fluoxetine affected normal 5-HT transport. Fluoxetine significantly prolonged the time constant for 5-HT-induced hSERT current. These results might be used to explain the long-lasting anti-anxiety effect of fluoxetine in clinical practice, because it increases the concentration of 5-HT in the synaptic cleft by its enduring suppression of the function of 5-HT transporters. **Conclusion:** Fluoxetine inhibits 5-HT reuptake by competing with 5-HT and changing the normal dynamics of hSERT.

Introduction

Neurotransmitter transporters play a very important role in terminating synaptic transmission. After the neurotransmitters are released into the synaptic cleft and activate the receptors, there are 3 ways to eliminate the function of neurotransmitters: (1) some neurotransmitters, such as acetylcholine (ACh), are decomposed by enzymes; (2) some are carried away by the blood; and (3) others, such as monoamine and amino acid transmitters, undergo reuptake by the transporters located in the plasma membrane of neurons[1]. Transporters are very interesting for 2 reasons: they succeed at an extraordinarily difficult job (pumping transmitter molecules across a membrane), and they are the molecular site at which many important psychoactive drugs act[2]. Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine transmitter, and it is mostly removed from the synaptic cleft by reuptake into the presynaptic membrane via the serotonin transporters (SERT). A low level of serotonin in the brain

is one of the causes of depression. The most popular antidepressant drugs are: (1) tricyclic compounds, such as imipramine, which block the reuptake of both norepinephrine and serotonin; (2) monoamine oxidase (MAO) inhibitors, such as phenelzine, which reduce the enzymatic degradation of serotonin and norepinephrine; (3) selective serotonin reuptake inhibitors (SSRI), which affect the presynaptic terminals.

Fluoxetine, an SSRI, acts only on the serotonin terminals[3] rather than on other monoamine transporters[4]. The clinical dosage of fluoxetine is much smaller than that of other antidepressant drugs, such as desipramine[5]. Fluoxetine elevates the levels of 5-HT in the brain, but its therapeutic actions take weeks to develop[6]. It is unknown why fluoxetine is so effective in blocking SERT and why it has a longer-lasting blocking effect than other antidepressant drugs, such as desipramine and paroxetine. Although many of the clinical pharmacological effects of fluoxetine have been well characterized in different species and by using different techni-

ques[5,7–9], little information is available regarding its electrophysiological properties. It is still unclear how fluoxetine reverses the 5-HT-induced currents.

By using human serotonin transporters (hSERT) heterologously expressed in Xenopus oocytes, and the 2-electrode voltage clamp technique, we studied the dynamic changes of hSERT in the process of transporting 5-HT under the influence of fluoxetine.

Materials and methods

Oocytes and cRNA Stage V and VI oocytes were harvested from adult *Xenopus* laevis females and prepared following the method of Machacak and Hartzell[10]. Human serotonin transporter (hSERT or 5-HTT) cRNA was transcribed from cDNA, which was cloned using a pOTV vector. The hSERT cDNA was donated by Vanderbilt University, Nashville,TN, USA. Stage V and VI defolliculated oocytes were injected with 52 nL (1 ng/nL) hSERT cRNA and then incubated at 19 °C in Ringer's solution (pH 7.6) supplemented with 1% penicillin-streptomycin stock and 5% horse serum. Oocytes were used 7–10 d after isolation and injection[11].

Electrophysiology Oocytes were voltage-clamped with 2 microelectrodes using an Axonpatch 200B amplifier (Axon Instruments, Foster city, USA) band-limited at 10 kHz. Microelectrodes were pulled using a programmable puller (Sutter Instruments, USA) such that their resistances were 1–2 MΩ when filled with 3 mol/L KCl solution and dipped into the bath solution. The bath solution was Na Ringer's solution, which comprised (in mmol/L): 96 NaCl, 2 KCl, 5 MgCl$_2$, 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 0.6 CaCl$_2$ (pH 7.6). A gravity pump operating at 1 mL/min was used to change the bath solution. Current records were stored on a computer and analyzed with the Origin 6 software package (Microcal Software). The holding potential was -50 mV, unless otherwise noted in the text or figure legends. All the data were obtained at room temperature (21–23 °C).

In the experiments, the oocytes were first perfused with Ringer's solution, then perfused with Ringer's solution containing 5-HT (10 µmol/L) to induce an inward current (5-HT-induced hSERT-current). Different concentrations of fluoxetine were added into the Ringer's solution containing 5-HT to reverse the 5-HT-induced currents. Finally, the perfusion solution was changed back to Ringer's solution. A fitted exponential function was used to obtain the time constants of the hSERT-currents.

Results

Reversal of 5-HT-induced hSERT-current by fluoxetine

5-HT at a concentration of 10 µmol/L was used to induce an inward current (Figure 1A). 5-HT-induced hSERT current can be reversed by fluoxetine (Figure 1B). The reversal time (τ3) of fluoxetine was much longer than the time taken for the 5-HT-induced hSERT current to reach its peak value (τ1) or the recovery time for the 5-HT-induced current to be washed out by Ringer's solution (τ2, Figure 1). The dissociation time constant of 5-HT-induced hSERT current and the reversal time constant of fluoxetine were 8.74 s and 23.56 s, respectively (Figure 1A, 1B). It seems that fluoxetine requires more time to reverse the function of hSERT than that required for 5-HT to combine with or dissociate from hSERT. If the oocytes were perfused with fluoxetine alone, there were no obvious inward or outward currents observed.
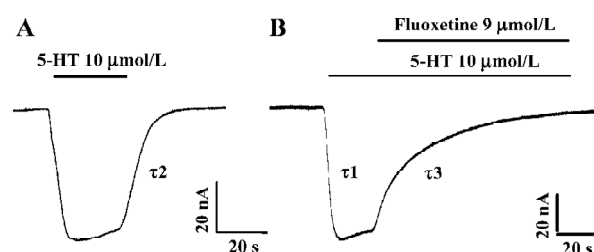

Figure 1. Fluoxetine reversed the 5-HT-induced hSERT current. The oocyte was first perfused with Ringer's solution. Then 10 µmol/L 5-HT and 9 µmol/L fluoxetine were added the solution successively as indicated by the bars. (A) 5-HT-induced inward hSERT-current. The dissociation time constant of 5-HT-induced hSERT current was 8.74 s (τ2). (B) The 5-HT-induced hSERT-current was reversed by fluoxetine. The time constant of 5-HT-induced hSERT current was 3.88 s (τ1). The inhibitive effect of fluoxetine on the hSERT current had a time constant of 23.56 s (τ3) in the present experiment. Voltage was held at -50 mV.

Concentration-dependent reversal of 5-HT-induced hSERT-current The mean percentage reversal effect of fluoxetine at different concentrations on hSERT-current were calculated. An exponential function was used to fit the curve. The fitted curve revealed the dose-dependent relationship between fluoxetine concentration and the percentage reversal of hSERT-current. Based on the fitted curve, RC$_{50}$ (50% reversal concentration) was 3.12 µmol/L (Table 1).

Reversal time constant *versus* the concentration of fluoxetine By using different concentrations of fluoxetine, we found that the time constants for reversing hSERT currents to steady state were dose-dependent (Figure 2). As the concentration of fluoxetine increased, the reversal time course became shorter. The time course shown in Figure 2A, using a concentration of 1 µmol/L, is much longer than those for concentrations of 3 and 6 µmol/L, as shown in

Table 1. Relationship between fluoxetine concentrations and the percentage reversal of hSERT currents (n=6–9). The percentage reversal of the hSERT current was used to evaluate the function of fluoxetine. Data are presented as mean±SE. Percentages are the hSERT current reversal divided by 5-HT-induced hSERT current, expressed as a percentage. For each concentration, 6–9 oocytes from different batches of frogs were studied.

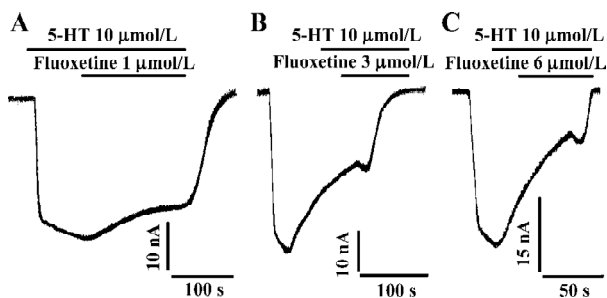| Fluoxetine concentration/μmol·L^{-1}	Percentage reversal of hSERT current /%
1	14.98±1.66
2	33.12±4.51
3	46.48±1.72
4	60.64±5.60
6	79.37±3.22
9	92.45±6.08
14	100.12±3.57

Figure 2. Different concentrations of fluoxetine reverse the hSERT currents at different speeds. The inhibition of hSERT current becomes faster as the concentration of fluoxetine increases.

Figure 2B and 2C, respectively. Table 2 shows the mean values and the standard errors of the time constants when the oocytes were treated with different concentrations of fluoxetine.

Table 2. Reversal time constants for inhibition of hSERT currents by fluoxetine (n=6–9). The reversal time constants are expressed as mean±SE. For each concentration, 6–9 oocytes from different batches of frogs were studied.

Fluoxetine concentration/μmol·L^{-1}	Time constant/s
1	75.81±6.78
2	57.39±5.83
3	40.76±3.98
4	33.58±2.54
6	30.46±2.61

Long-lasting effect of fluoxetine on the hSERT When the 5-HT-induced hSERT current was first reversed with fluoxetine, then washed away with Ringer's to the control level, and later the same perfusion protocol was repeated, we found that on the second perfusion with the same concentration of 5-HT, the time constant for 5-HT-induced current became much greater than that for the first perfusion (Figure 3). Relative to the first perfusion, the time constant for 5-HT-induced hSERT current increased from 4.72 s to 83.68 s (Figure 3). But for both perfusions, there was no difference in the reversal effect of fluoxetine on the hSERT current (Figures 3 and 4): the reversal time constants were 25.32 s and 26.97 s, respectively. Our results also indicated that the long-lasting effect of fluoxetine was dose-dependent (Table 3).
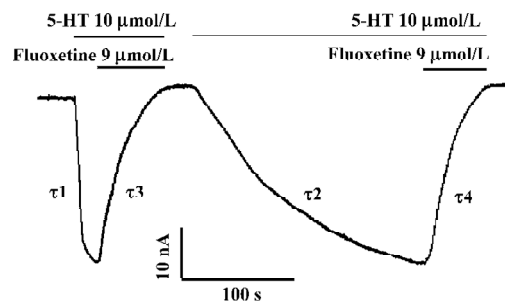

Figure 3. Long-lasting effect of fluoxetine on hSERT. The protocol shown in the figure was performed on the same oocyte repeatedly. During the first treatment, the 5-HT-induced hSERT current had a smaller time constant (τ1= 4.72 s) than during the second treatment (τ2=83.68 s). However, the time constants of fluoxetine-inhibited hSERT currents were similar (τ3=25.32 s, τ4=26.97 s). It seems that fluoxetine has a long-lasting effect on hSERT.

Table 3. Fluoxetine pretreatment changes the time constant for the occurrence of 5-HT-induced current (n=4–6). τ1 was the time constant for 5-HT-induced hSERT current (as shown in Figure 3), which served as the control. τ2 was the time constant for 5-HT-induced hSERT current when the oocyte was treated again with the same perfusion protocol (Figure 3). ^{c}P<0.01 *vs* τ1.

Fluoxetine concentration/μmol·L^{-1}	τ1/s	τ2/s
3		64.81±1.69^{c}
4	5.35±0.47	76.76±5.40^{c}
6		86.52±4.11^{c}

Discussion

Before performing the experiment with fluoxetine, we first had to prove that the 5-HT-induced current was hSERT cur-

rent[11]. 5-HT is co-transported with Na^+ and Cl^- ions. If the holding potentials in the voltage clamping are changed, with a constant concentration of perfused 5-HT, the amplitude of the current should change accordingly. We observed that the more negative the holding potential used, the larger the inward current, which demonstrated that this was a voltage-dependent current. Furthermore, the 5-HT-induced current could be totally blocked by desipramine, a specific blocker of the monoamine transporter (data not shown), which also confirmed that this current was heteroexpressed hSERT-current.

After the 5-HT-induced current reached a stable state, fluoxetine was added into the perfusion solution. The 5-HT-induced current then became smaller and smaller (Figure 1B), and finally the current trace returned to zero, which indicated that the 5-HT-induced current was almost completely blocked by fluoxetine at a concentration of 9 μmol/L.

We found that the blocking effect of fluoxetine was not as fast as we expected. The blocking time ($\tau 3$) was much longer than the time needed for 5-HT-induced current to reach its maximum value ($\tau 1$; Figure 1), because the time constant for the former was 23.56 s and for the later it was 3.88 s.

The 5-HT current could be dose-dependently reversed by fluoxetine; the RC_{50} was approximately 3.12 μmol/L. The reversal time constant varied with different concentrations of fluoxetine (Table 2). The higher the concentration of fluoxetine, the faster the reversal process.

We observed that the reversal effects of fluoxetine are characterized by 3 main features:

First, Compared with the time course from washing out the 5-HT with control Ringer's solution (Figure 1A, 1B), the reversal time constant of fluoxetine was much longer than those produced by Ringer's solution, which suggests that fluoxetine is competing with 5-HT for the binding sites in the transporter, resulting in a decreased current. However, the molecular binding site of fluoxetine needs further investigation.

Second, when the hSERT-expressing oocyte was repeatedly perfused with fluoxetine and then washed out with Ringer's solution, the time constant for the occurrence of 5-HT current during the second or third perfusion became much greater than that for the first perfusion ($\tau 2 > \tau 1$; Figure 3) and even greater than the reversal time constant of fluoxetine ($\tau 2 > \tau 4$). It has been reported that fluoxetine has high affinity to hSERT ($K_i = 33$ nmol/L)[13] . This could explain why fluoxetine had a long-lasting effect on hSERT ($\tau 2 > \tau 1$; Figure 3) in our experiments.

Third, all reversal processes, no matter what concentration of fluoxetine was used, could be fitted with a single exponential function. The exponential function $B = B_0 + B_1 e^{-k1t} + B_2 e^{-k2t} + ...$ allowed us to calculate the combination or dissociation rate constants of receptor-ligand complexes and speculate regarding the dynamic mechanisms involved[12]. The time course of the current reversals reflected the combination of fluoxetine with hSERT. The single exponential function fitting suggests that fluoxetine reverses the 5-HT-induced-current by competing with the binding of 5-HT to hSERT. Other mechanisms of antidepressant therapy[6], such as regulating the density of hSERT, activating neurotrophins or glucocorticoids in the cells, and modulating the intracellular signal pathways, may not be involved in our experiments. If they had been, there might have been more than one time constant observed.

In clinical practice, fluoxetine is used to treat patients who have reduced amounts of 5-HT in the brain. Because fluoxetine can suppress the function of 5-HT transporters, it would leave more 5-HT in the synaptic clefts. Its long-lasting effect on hSERT might explain why patients take much more desipramine (up to 200 mg/d) than fluoxetine (20 mg/d) in clinical treatments[5]. The lower fluoxetine dosage used could reduce the occurrence of side-effects such as fever, headache, naupathia, diarrhea, and dyspnea.

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Info: Ms Xiao-dan ZHAO
           Chinese Pharmacological Society
           1, Xian Nong Tan St
           Beijing 100050, China
           Phn/Fax 86-10-6316-5211
           E-mail zhxd@imm.ac.cn
           Http://www.iuphar2006.org/
           Http://www.cnphars.org/