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INTERACTION OF FLUOXETINE WITH THE HUMAN PLACENTAL SEROTONIN TRANSPORTER

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Abstract—The interaction of fluoxetine, a non-tricyclic antidepressant, with the human placental serotonin transporter was investigated by studying its influence on [^{3}H]paroxetine binding to the transporter and on [^{3}H]serotonin uptake via the transporter. These studies were done using brush-border membrane vesicles purified from normal term human placentas. Fluoxetine inhibited binding of paroxetine to the membrane vesicles in a concentration-dependent manner, with a K_i value of 3 nM. Kinetic analysis revealed that the inhibition was competitive because the presence of 10 nM fluoxetine increased the K_d for paroxetine from 72 to 461 pM, but had no effect on the B_{max}. Fluoxetine also caused a time-dependent dissociation of paroxetine already bound to the transporter. The dissociation followed first-order kinetics. Uptake of serotonin in these membrane vesicles was also inhibited by fluoxetine. The inhibition was concentration dependent with a K_i value of 66 nM at pH 7.5 and 80 nM at pH 6.5. The effect of fluoxetine on the uptake kinetics was to increase the apparent dissociation constant (K_i) for serotonin without influencing the maximal transport capacity (V_{max}). The results demonstrate that fluoxetine is a high-affinity ligand and a potent inhibitor of the serotonin transporter found in the human placental brush-border membrane.

Preclinical studies on the characterization of tricyclic and non-tricyclic antidepressants which inhibit serotonin uptake have primarily been done using synaptosomal fractions from the brains of laboratory animals [1, 2]. Postmortem human brains have been used only to a limited extent for this purpose [3–6]. After the discovery that the plasma membrane of platelets also possesses an active serotonin transport system similar to the one described in the synaptosomal membrane [1, 7–9], the platelet plasma membrane has become a much-used experimental tool to study the binding and inhibition parameters of serotonin uptake inhibitors. However, there are prohibitive factors which make the use of human platelets for serotonin uptake studies less than ideal, if not impossible. Fresh human platelets are expensive, and the yield of plasma membrane from platelets is small. Purity of the plasma membrane preparation from platelets is very critical because contamination of storage granules, which are known to contain an active serotonin transport system distinct from that of the plasma membrane [10], will make interpretation of the data difficult. Owing to these reasons, extensive studies on serotonin uptake using human platelet plasma membrane vesicles are costly and time-consuming. Outdated human platelets have been used in some cases, but one must be cautious in the interpretation of the data because some of the characteristics of the transporter may have been compromised during the long storage. It is clear from the foregoing discussion that identification of another human tissue which is easily obtainable and which possesses serotonin transport activity will be highly desirable and extremely useful in screening for the efficacy of serotonin uptake inhibitors and also in delineating the underlying mechanism of their inhibition.

Recently, a high-affinity serotonin transport system was described in our laboratory in brush-border membrane vesicles prepared from the syncytiotrophoblast of normal human term placentas [11, 12]. We have shown this system to be kinetically similar to that of the platelet plasma membrane [13]. Antidepressants such as imipramine and paroxetine interact with the placental serotonin transporter with high affinity. These studies have suggested that the human placental serotonin transporter would possibly qualify as an alternate model system for identification and characterization of drugs which may have an effect on serotonin uptake. Additionally, though the role of placental serotonin transport in growth and development of the fetus is unknown at the present time, the presence of the transport system in the placenta indicates that future drug tests should ascertain the degree to which a drug will or will not affect placental serotonin transport.

The purpose of the present investigation was to characterize the interaction of the placental brush-border membrane serotonin transporter with fluoxetine, one of the serotonin uptake inhibitors. Fluoxetine [3 - (*p* - trifluoromethylphenoxy) - *N* - methyl-3-phenylpropylamine] is a nontricyclic antidepressant, which is effective in relieving major depression [14–17] and is being considered for the treatment of obesity [18–20] as well as bulemic patients [21]. Fluoxetine, like paroxetine, is reported to have little affinity for neuronal receptors and to be highly selective for inhibition of serotonin uptake [17, 22].

MATERIALS AND METHODS

Materials. 5-[1,2-^{3}H(n)]Hydroxytryptamine binoxalate (specific radioactivity, 30.4 Ci/mmol) and

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[*phenyl*-6-^{3}H]paroxetine (specific radioactivity, 26.5 Ci/mmol) were purchased from DuPont-New England Nuclear. Serotonin and imipramine were obtained from Sigma. Fluoxetine was a gift from the Eli Lilly Co. All other chemicals were of analytical grade.

Preparation of human placental brush-border membrane vesicles. Brush-border membrane vesicles were prepared from normal human term placentas by the Mg^{2+}-aggregation method as described previously [23, 24]. For the uptake studies, the membrane vesicles were washed with either 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes)/Tris buffer, pH 7.5, containing 300 mM mannitol or 20 mM 4-morpholine ethanesulfonic acid (Mes)/Tris buffer, pH 6.5, containing 300 mM mannitol. For the binding studies, the membrane vesicles were washed with 50 mM Tris/HCl buffer, pH 7.4, containing 120 mM NaCl and 5 mM KCl. Protein concentration in final preparations was determined according to the method of Lowry *et al.* [25] using bovine serum albumin as the standard. The membrane vesicles (protein concentration, 5 mg/mL) were then stored in small aliquots in liquid N_2 until used.

Serotonin uptake assay. [^{3}H]Serotonin uptake measurements were made using a rapid filtration technique as previously described [26]. Millipore filters (DAWP, 0.65 μm pore size) were used in these experiments. Uptake was initiated by mixing 40 μL of the membrane suspension with 160 μL of appropriate uptake buffer containing [^{3}H]serotonin. The composition of the uptake buffer was either 20 mM Hepes/Tris, 150 mM NaCl, pH 7.5, or 20 mM Mes/Tris, 150 mM NaCl, pH 6.5. After incubation for a desired time at room temperature (21–22°), uptake was terminated by adding 3 mL of ice-cold stop buffer (160 mM KCl buffered with either 5 mM Hepes/Tris, pH 7.5, or 5 mM Mes/Tris, pH 6.5), and the mixture was filtered. The filter was washed with 3 × 5 mL of stop buffer, and the radioactivity associated with the filter was counted by liquid scintillation spectrometry. Serotonin uptake which occurred specifically via the serotonin transporter was calculated by subtracting the uptake measured in the presence of 10 μM imipramine from the total uptake measured in the absence of imipramine.

Paroxetine binding assay. Binding of [^{3}H]paroxetine to the brush-border membrane vesicles was assayed by a rapid filtration technique described by Mellerup *et al.* [27] with a few modifications. Whatman GF/F glass fiber filters (0.7 μm pore size) were used in these experiments. Binding assay was done in a total volume of 1 mL containing 50 mM Tris/HCl (pH 7.4), 120 mM NaCl, 5 mM KCl and a desired concentration of [^{3}H]paroxetine. One hundred micrograms of membrane protein was used for each assay. Binding was carried out at room temperature for 60 min after which 3 mL of ice-cold binding buffer was added and the mixture rapidly filtered. The filter was washed with 3 × 5 mL of binding buffer, and the radioactivity associated with the filter was counted by liquid scintillation spectrometry. Specific binding was calculated by subtracting the binding measured in the presence of 100 μM imipramine from the total binding measured in the absence of imipramine.

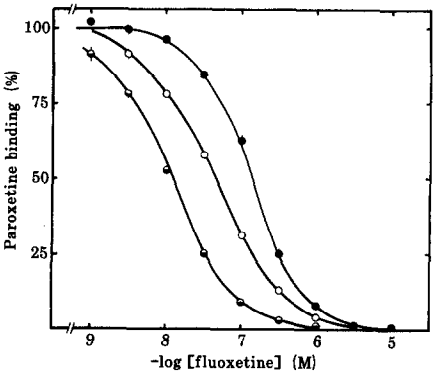

Fig. 1. Concentration-dependent inhibition of [^{3}H]paroxetine binding by fluoxetine. Binding of 3 nM (●), 1 nM (○) and 0.25 nM (◒) [^{3}H]paroxetine to placental brush-border membrane vesicles was determined at 22° in the absence and presence of various concentrations of fluoxetine. In the absence of fluoxetine, the specific paroxetine binding was 4.17 ± 0.34, 4.48 ± 0.07 and 1.78 ± 0.03 pmol/mg of membrane protein at 3 nM, 1 nM and 0.25 nM paroxetine respectively. These values were taken as 100% for respective concentrations of paroxetine. Values are means ± SE (N = 4; two membrane preparations). When not shown, the standard error is within the symbol.

Statistics. Each experiment was carried out in duplicate or triplicate using 2–5 different membrane preparations. Statistical analysis of the experimental data was done using the computer statistics package, Statgraphics (STSC, Rockville, MD). For the analysis of non-linear graphs, a custom-made computer program (MCG Fit) was used. The results are given as means ± SE.

RESULTS

Concentration-dependent inhibition of [^{3}H]paroxetine binding by fluoxetine. We used two approaches to investigate the interaction of fluoxetine with the human placental serotonin transporter. In the first approach, the influence of fluoxetine on the binding of paroxetine to the placental brush-border membrane vesicles was investigated. Paroxetine is a specific ligand for the serotonin transporter present in the platelet plasma membrane [27] and brain synaptosomal membrane [5, 28]. We have shown recently that the placental serotonin transporter also interacts specifically with this ligand [29]. Therefore, we have evaluated the inhibition of [^{3}H]paroxetine binding by fluoxetine by measuring the binding of paroxetine at three different concentrations (0.25, 1 and 3 nM) to the placental brush-border membrane vesicles in the presence of increasing concentrations of fluoxetine over a range of 10^{-9}–10^{-5} M (Fig. 1). The IC_{50} values calculated for fluoxetine at the above-mentioned three concentrations of paroxetine were 13, 42, and 145 nM respectively. The inhibition

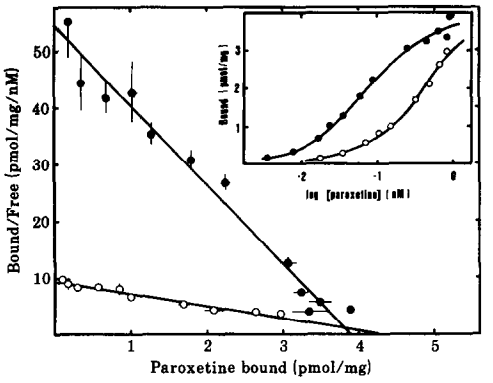

Fig. 2. Effect of fluoxetine on the kinetics of paroxetine binding. Binding of [^{3}H]paroxetine to placental brush-border membrane vesicles was determined at various concentrations of paroxetine (range, 0.0125 to 1.125 nM) in the absence and presence of 10 nM fluoxetine. The data, given as a Scatchard plot, represent means ± SE. When not shown, the standard error is within the symbol. Inset: binding isotherm of the same data. Key: (●——●) control (N = 18, five membrane preparations), and (○——○) fluoxetine (N = 4, two membrane preparations).

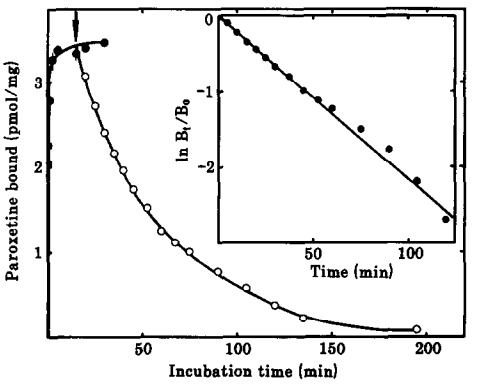

Fig. 3. Equilibrium dissociation of paroxetine by fluoxetine. Binding of 3 nM [^{3}H]paroxetine to placental brush-border membrane vesicles was initiated in the absence of fluoxetine and allowed to go to equilibrium. At 15 min, a stock solution of fluoxetine was added to the incubation mixture to a final concentration of 10 μM (arrow). Aliquots were removed from the mixture at indicated time intervals to determine the amount of [^{3}H]paroxetine still bound to the membranes. Values are means ± SE (N = 4, two membrane preparations). When not shown, the standard error is within the symbol. The dissociation rate constant (K_{-1}) was calculated from the slope of the plot, ln B_t/B_0 versus time (inset).

constant (K_i) was calculated using the formula

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

where $[L]$ is the concentration of paroxetine and K_d is the dissociation constant for the binding of paroxetine to the membrane vesicles [30]. The K_d for paroxetine binding was 0.072 nM (see below). The K_i values thus determined were similar at all three concentrations of paroxetine (2.9, 2.8, and 3.4 nM, respectively, with a mean value of 3 nM).

Effects of fluoxetine on the kinetics of paroxetine binding. The binding parameters (K_d and B_{max}) for paroxetine binding to the placental brush-border membrane vesicles were determined in the presence and absence of fluoxetine. In these experiments, specific binding of [^{3}H]paroxetine to the membrane vesicles was determined over a concentration range of 0.0125 to 1.125 nM. The concentration of fluoxetine when present was 10 nM. Figure 2, inset, describes the binding isotherms for paroxetine in the absence and presence of fluoxetine. When these data were subjected to Scatchard analysis (bound versus bound/free), linear plots were obtained indicating the presence of a single class of binding sites. The primary influence of fluoxetine on the binding of paroxetine was to reduce the affinity of the transporter for paroxetine. The K_d value calculated in the absence of fluoxetine was 72 ± 0.4 pM which increased 6-fold to 461 ± 32 pM in the presence of 10 nM fluoxetine. The maximal binding capacity, B_{max}, remained unchanged (3.9 ± 0.1 and 4.3 ± 0.2 pmol/mg of protein in the absence and presence of fluoxetine respectively).

Dissociation of bound [^{3}H]paroxetine by fluoxetine. We further analyzed the interaction of the placental serotonin transporter with fluoxetine by investigating the ability of fluoxetine to dissociate paroxetine which was already bound to the transporter. The binding of [^{3}H]paroxetine to placental brush-border membrane vesicles was very rapid with maximal binding occurring within 5 min at room temperature. Figure 3 shows the time-dependent [^{3}H]paroxetine binding and also the rapid dissociation of [^{3}H]-paroxetine upon addition of 10 μM fluoxetine at equilibrium (15 min). The dissociation rate constant, K_{-1}, of [^{3}H]paroxetine binding was calculated from the equation $\ln(B_t/B_0) = -K_{-1} \cdot t$ where B_t is the specific binding of [^{3}H]paroxetine at time t and B_0 is the specific binding of [^{3}H]paroxetine at time zero [31]. A plot of $\ln(B_t/B_0)$ versus t in the presence of fluoxetine was linear (Fig. 3, inset; $r = -0.996$) with a slope of 0.021 ± 0.001 (K_{-1}).

We also determined the association rate constant, K_{+1}, of [^{3}H]paroxetine binding from the time-dependent binding of [^{3}H]paroxetine to the placental brush-border membrane vesicles (Fig. 4A). K_{+1} was calculated from the equation $\ln(B_0/B_0 - B_t) = ([L]K_{+1} + K_{-1})t$ where B_0 is the specific binding of [^{3}H]paroxetine at equilibrium (15 min), B_t is the specific binding of [^{3}H]paroxetine at time t, and $[L]$ is the concentration of [^{3}H]paroxetine [31]. The concentration of [^{3}H]paroxetine used in these experiments was 3 nM. The plot of $\ln(B_0/B_0 - B_t)$ versus t was linear (Fig. 4B; $r = 0.999$) with a slope of 1.16 ± 0.02. This slope was equal to $[L]K_{+1} + K_{-1}$. By substituting the values for $[L]$ (3 nM) and K_{-1} (0.021), the value for K_{+1} was calculated to be 0.38. From these values of association and dissociation rate constants, the K_d for [^{3}H]paroxetine binding was calculated using the equation $K_d = K_{-1}/K_{+1}$. The value for K_d was 55 pM which was very close to the K_d value calculated by Scatchard analysis.

Concentration-dependent inhibition of serotonin uptake by fluoxetine. The second approach we used

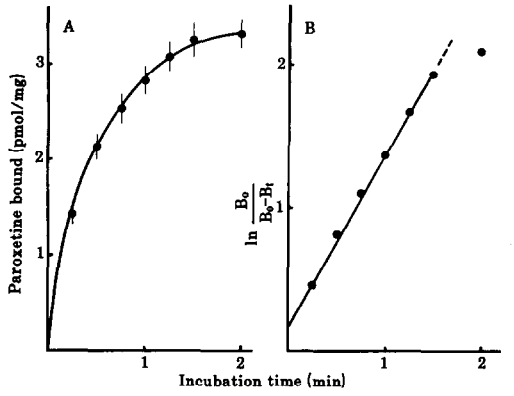

Fig. 4. Association kinetics for the binding of paroxetine. Binding of 3 nM [^{3}H]paroxetine to placental brush-border membrane vesicles was determined. (A) Time course of paroxetine binding. Values are means ±SE (N = 3, two membrane preparations). (B) A plot of $\ln(B_0/B_0 - B_t)$ versus time from the same data.

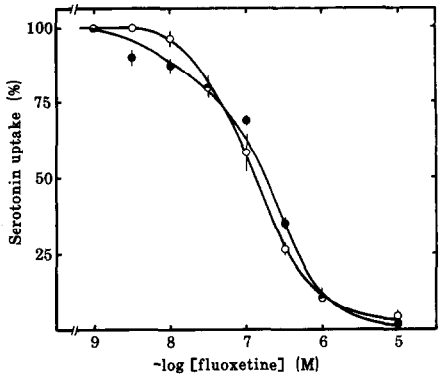

Fig. 5. Concentration-dependent inhibition of [^{3}H]serotonin uptake by fluoxetine. Uptake of 25 nM [^{3}H]serotonin into placental brush-border membrane vesicles was determined with a 15-sec incubation in the presence of an inwardly directed NaCl gradient and at two different pH values, 6.5 (○——○) and 7.5 (●——●). The concentration of fluoxetine was varied between 10^{-9} and 10^{-5} M. In the absence of fluoxetine, the value for serotonin uptake was 3.24 ± 0.03 pmol/mg protein/15 sec at pH 6.5 and 1.74 ± 0.16 pmol/mg protein/15 sec at pH 7.5. These values were taken as 100% at respective pH. Values are means ± SE (N = 4, two membrane preparations). When not shown, the standard error is within the symbol.

to investigate the interaction of fluoxetine with the human placental serotonin transporter was to evaluate the influence of this ligand on serotonin uptake in placental brush-border membrane vesicles. The uptake of serotonin in these vesicles is driven by an inwardly directed NaCl gradient [11, 12]. In addition, this NaCl gradient-dependent uptake is influenced markedly by pH [13]. The presence of an inside-acidic pH, with or without a transmembrane pH gradient, greatly stimulates the NaCl-dependent serotonin uptake. We therefore investigated the effects of increasing concentrations of fluoxetine over a range of 10^{-9}–10^{-5} M on the initial rates of serotonin uptake in the presence of a NaCl gradient ($[NaCl]_0 = 120$ mM; $[NaCl]_i = 0$) at two pH values, 6.5 and 7.5. At both pH values, fluoxetine was found to be a potent inhibitor of serotonin uptake (Fig. 5). Fluoxetine inhibited serotonin uptake with an IC_{50} of 174 nM at pH 7.5. The corresponding value at pH 6.5 was 132 nM. The K_i values calculated using the K_t values for serotonin uptake (15 nM at pH 7.5 and 38 nM at pH 6.5) and the concentration of serotonin employed in uptake measurements (25 nM) were 66 and 80 nM at pH 7.5 and 6.5 respectively.

Effects of fluoxetine on the kinetic parameters of serotonin uptake. The initial rates of serotonin uptake (15 sec incubation) were measured at pH 6.5 in the presence of a NaCl gradient over a serotonin concentration range of 20–200 nM in the presence and absence of 100 nM fluoxetine. Eadie–Hofstee analysis (initial uptake rate/serotonin concentration versus initial uptake rate) gave linear plots ($r^2 > 0.98$) indicating the presence of a single, saturable, transport system (Fig. 6). The effect of fluoxetine on the serotonin transporter was to decrease the affinity of the transporter for serotonin because the apparent dissociation constant (K_t, the concentration of substrate at which the uptake rate is equal to one-half of the maximal velocity) was 38 ± 2 nM in the absence of fluoxetine but the value increased to 87 ± 6 nM in the presence of fluoxetine. The maximal velocity, V_{max}, however, remained unchanged

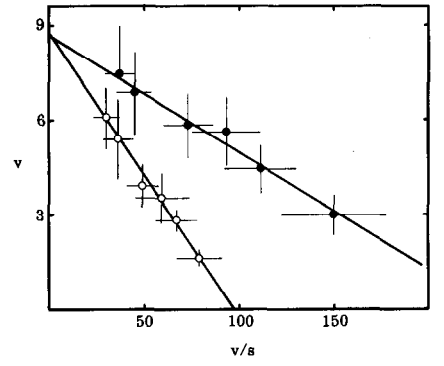

Fig. 6. Effect of fluoxetine on the kinetics of serotonin uptake. Uptake of [^{3}H]serotonin into placental brush-border membrane vesicles was determined with a 15-sec incubation at pH 6.5 in the presence of an inwardly directed NaCl gradient. The concentration of serotonin was varied between 20 and 200 nM. Values are means ± SE (N = 6, three membrane preparations). v, initial uptake rate of serotonin in pmol/mg protein/15 sec; s, serotonin concentration in μM. Key: (●——●) control, and (○——○) 100 nM fluoxetine.

(8.7 ± 0.2 and 8.6 ± 0.3 pmol/mg protein/15 sec in the absence and presence of fluoxetine respectively).

DISCUSSION

The human placenta is an easily obtainable tissue. The presence of a high-affinity serotonin transport system in the human placental brush-border membrane which binds tricyclic as well as non-tricyclic antidepressants makes this tissue an excellent experimental tool to investigate drug interactions with the

serotonin transporter. The current investigation was aimed at characterizing the placental serotonin transporter with respect to its potential as a model for antidepressant drug binding and serotonin uptake inhibition. In the present study, the interaction of fluoxetine with the transporter was evaluated by studying the influence of this compound on the binding of [^{3}H]paroxetine as well as on the uptake of [^{3}H]serotonin using purified human placental brush-border membrane vesicles.

The results of this investigation indicate that fluoxetine interacts with the placental serotonin transporter with high affinity as evidenced by reduced serotonin uptake and reduced paroxetine binding in the presence of this compound. The K_i values to inhibit serotonin uptake and paroxetine binding were 66 and 3 nM respectively. Kinetic analyses revealed that fluoxetine inhibition of serotonin uptake as well as paroxetine binding was competitive in nature. Moreover, fluoxetine was able to displace paroxetine which was already bound to the transporter. The equilibrium displacement strictly followed first-order kinetics. This enabled us to determine the dissociation rate constant for the binding of paroxetine to the transporter. The K_d value for paroxetine binding calculated using this dissociation rate constant was very close to the value determined by Scatchard analysis (55 vs 72 pM). All these data suggest that fluoxetine and paroxetine interact with a single binding site on the transporter. Kinetic data suggest that serotonin also interacts with the same site. The K_i values determined for fluoxetine to inhibit serotonin uptake and paroxetine binding differed at least by an order of magnitude. However, this was expected because, unlike paroxetine binding, serotonin uptake via the transporter is a complex process which includes, in addition to the binding of serotonin to the ligand binding site, two other steps namely translocation of the serotonin–transporter complex across the membrane and coupling of this translocation to the transmembrane NaCl gradient. The measured uptake rate represents a combination of the individual rates of these various events. The K_i value for fluoxetine to inhibit serotonin uptake describes the influence of fluoxetine on the whole process rather than on any single event. Therefore, the observed difference in the K_i values for fluoxetine to inhibit serotonin uptake and paroxetine binding does not constitute evidence against our conclusion that all three ligands, serotonin, paroxetine and fluoxetine, interact with a single binding site on the transporter.

The antidepressants which inhibit serotonin uptake are divided into two structurally distinct groups, tricyclic (e.g. imipramine and desipramine) and non-tricyclic (e.g. paroxetine and fluoxetine). There is strong supporting evidence that the inhibitors belonging to each group interact with the serotonin transporter in a mutually exclusive manner [32, 33]. But, there is a considerable debate regarding whether or not the binding site is common for the two groups of inhibitors [6, 32–36]. However, it appears that the recently observed heterogeneity of imipramine binding sites in the neuronal tissue may have been responsible, at least to some extent, for the previous conclusions supporting the existence of distinct binding sites for tricyclic and non-tricyclic inhibitors [37–39]. The placental serotonin transporter has so far been characterized only with respect to the binding of non-tricyclic inhibitors such as paroxetine [29] and fluoxetine [present study]. Imipramine and desipramine, tricyclic inhibitors, have been shown to inhibit serotonin uptake [11] as well as paroxetine binding [29] in placental brush-border membrane vesicles, but the binding characteristics of this class of inhibitors have not been investigated. It will be necessary, therefore, to compare the binding properties of tricyclic and non-tricyclic inhibitors in the placental membrane preparation determined under identical experimental conditions before one can answer the question whether or not the two groups of inhibitors bind to the same site on the placental serotonin transporter.

The characteristics of serotonin transporter have been studied in great detail in the platelet and the brain. Even though there are a number of similarities between the serotonin transporters in these two tissues, recent work has indicated that these transporters may not be identical as was once believed [40–43]. Important differences between the platelet and the neuronal transporters include Na^+ : serotonin stoichiometry and modulation of the transport function by pH and transmembrane pH gradients. Our studies with the placental serotonin transporter [12, 13] strongly suggest that this transporter resembles more closely the platelet transporter than the neuronal transporter. The serotonin transporters of the platelet and the placenta are stimulated markedly by an inside-acidic pH with or without a transmembrane pH gradient, whereas the serotonin transporter of the brain is not stimulated but rather inhibited by an acidic pH. The possibility that the placental serotonin transporter may be distinct from the neuronal serotonin transporter makes the results of the present study interesting and important because previous work on the interaction of fluoxetine with the serotonin transporter has been done predominantly with the brain tissue [5, 44, 45]. Fluoxetine inhibits serotonin uptake and paroxetine binding in the brain with a K_i value of 25–52 nM and 0.9 nM respectively. A comparison of these values with corresponding values obtained in the present study indicates that the sensitivity of the placental serotonin transporter to fluoxetine is 2–3 times less compared with the neuronal serotonin transporter.

In conclusion, the present study shows that fluoxetine was a potent inhibitor of the human placental serotonin transporter. This inhibition was demonstrable with respect to serotonin uptake as well as binding of paroxetine in isolated placental brush-border membrane vesicles.

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