Fluoxetine-induced alterations in human platelet serotonin transporter expression: serotonin transporter polymorphism effects

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Introduction

The serotonin transporter (SERT) is the target molecule for most of the antidepressant drugs currently in use — both the older tricyclic drugs and the newer selective serotonin reuptake inhibitors (SSRI). When these drugs bind to the SERT, they inhibit its normal function — the uptake of serotonin (5-hydroxytryptamine [5-HT]) from the synapse, leading to...
an accumulation of 5-HT in the synapse. This short-term effect is believed to be critical in causing eventual clinical improvement, although the entire mechanism responsible has not been elucidated. In addition to acute blockade, it is possible that changes may be induced in SERT function after prolonged exposure to antidepressant drugs. Recent studies employing sensitive measures of SERT expression over extended time periods have found that antidepressant exposure decreases SERT levels in the rat midbrain. This delayed long-term regulatory effect eventually has a much greater impact on actual synaptic levels of serotonin than the initial pharmacological blockade. It remains unclear whether such marked adaptive effects occur in humans.

Progressively diminishing SERT concentrations could decrease the SERT’s ability to remove 5-HT from the synapse, thus continually increasing synaptic 5-HT concentrations and perhaps also damping temporal fluctuations. Alterations in SERT function could also have an effect on neuronal membrane potential, because ionic currents pass through the SERT during translocation of 5-HT, as well as during apparent resting intervals. From a clinical perspective, if further regulation of SERT function occurs in addition to simple uptake blockade, such a process could explain the extended time necessary for treatment response, as well as side effects that develop during the course of treatment. Because of these considerations, regulation of SERT function could be an important aspect of the clinical response to longer-term antidepressant exposure.

The present experiments evaluated SERT expression levels in healthy control platelets, harvested and maintained alive for 24 hours and treated with fluoxetine, to test the hypothesis that fluoxetine exposure would alter total SERT immunoreactivity (IR). We chose to study platelets because they express large numbers of SERT molecules and they can be obtained in blood samples. Further, the number and/or function of SERT molecules expressed in the human brain or peripherally may be influenced by a common polymorphism in the SERT promoter region (human serotonin transporter coding [5-HTTLPR]), although there are also reports showing no relation. Several studies have suggested that the genotype for the SERT polymorphism is related to eventual antidepressant response. For these reasons, we determined the SERT promoter genotype in the study subjects to discover whether it affected fluoxetine-related regulation of platelet SERT expression.

Methods

Twenty-one subjects were studied after providing informed consent, as approved by the Human Studies Committee of the Ann Arbor Veteran’s Affairs Medical Center. Subjects were under 50 years of age (range 22–48 yr) and included 3 women and 18 men. No subject had a history of taking antidepressant medication.

Platelet preparation

Platelets were harvested in a vacutainer tube containing sodium citrate (0.1 mM). An initial platelet-rich supernatant was obtained through mild centrifugation (160 ∞ g for 10 min), followed by platelet separation with more intense centrifugation (12 000 ∞ g for 10 min). The resulting pellet was washed twice for 10 minutes in phosphate buffer solution (PBS). The platelets were resuspended in PBS. One-half of each subject’s platelets were treated with fluoxetine, 0.1 mM in PBS, and the other one-half were treated with PBS only, for 24 hours on a vortexer at 4°C, to decrease nonspecific degradation. The concentration of fluoxetine used was similar to serum levels found in human patients. After incubation, platelets were centrifuged 12 000 ∞ g for 10 minutes, then lysed with radioimmunoprecipitation buffer with protease inhibitors and agitated for 30 minutes at 4°C. Total protein concentrations were determined spectroscopically with the Bio-Rad DC protein assay kit; individual gel loadings (further described below) were based on these determinations. Total protein concentrations were virtually identical between treated and untreated samples, indicating that treatment did not cause nonspecific degradation of total protein. An initial time course study found that preserving platelets for periods longer than 24 hours resulted in declining [3H]5-HT uptake rates (Fig. 1). One tube of frozen blood was sent for genotyping by the 5-HTTLPR (see below). At least 5 subjects were identified for each of the 3 genotypes.

[3H]5-HT uptake assays

We assessed platelet uptake to determine whether the SERT remained functional during the 24-hour treatment period. Krebs Phosphate (KP) buffer (pH 7.4) was used and contained NaCl 120 mM/L, KCl 4.8 mM/L, MgSO4 1.4 mM/L, Na2HPO4 16 mM, glucose 11 mM/L, ascorbic acid 1.0 mM/L, pargyline 0.03 mM/L and CaCl2 1.2 mM/L. Cells were incubated at room temperature for 30 minutes to stabilize in KP buffer before assay. [3H]5-HT (27.5 µM) was added to each of 3 replicate tubes and the platelets were maintained in the KP buffer for 30 minutes to stabilize in KP buffer before assay. [3H]5-HT uptake was determined as described below.

Fig. 1: Effect of platelet storage time on [3H]5-hydroxytryptamine uptake. There was a noticeable decline by the second day; thus additional experiments were performed within 24 hours. Each data point represents the results from 3 replicate tubes. The error bars display standard deviations. vMAX = maximal update velocity.
were dissolved in 500 μL 1% sodium dodecyl sulfate (SDS), and the solution was counted, as described above. Nonspecific uptake was determined in the presence of 10 μM (-)imipramine.

SERT IR quantification

SERT levels were measured by Western blot assays of SERT IR, with a method similar to that used with the dopamine transporter. This was done in preference to measuring radiolabelled serotonin uptake, or radioligand binding, because each of these would require the complete washing out of inhibitory levels of fluoxetine to below the nanomolar range, which could be difficult to accomplish without losing substantial amounts of platelets. After treatment incubation and total protein concentrations were determined, platelet protein samples (0.25 mg/gel lane, based on film response standard curve; see Fig. 2) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 7.5% polyacrylamide solution (8 5 cm, 2 mm thick). Samples were then electrophoretically transferred to Immobilon-P membranes at 36 mA for 1 hour. Blots were incubated with 5% (w/v) dry milk in Tris-buffered saline with Tween (TTBS) 0.1% Tween 20, 0.15 M NaCl, and 10 mMol/L Tris-HCl, pH 7.4) for 1 hour. Each step was performed at room temperature. The primary antibody used was a rabbit anti-human serotonin transporter antibody to 1:4000 dilution (Chemicon, Inc, Temecula, Calif.), followed by a horseradish peroxidase-conjugated donkey anti-rabbit IgG second antibody (Amersham Life Sciences, Piscataway, NJ). The antigen-antibody complex was detected with the ECL plus Western blotting detection system at 1:2000 dilution (Amersham Life Sciences, Piscataway, NJ). An intense array of bands were varied to ensure that the dynamic range of the film was used. The relative optical densities of protein bands were digitized and quantitated with a microcomputer imaging analysis system (Microcomputer Imaging Devices, Ottawa, Ont.), and the results were expressed as a percentage change, compared with the vehicle-treated platelets, and analyzed with a 1-way analysis of variance (ANOVA) to test for genotype effect. No absolute measure of basal SERT levels was performed, because we have previously described the relation between SERT expression in the human brain and in platelets.

SERT genotyping

We used the Pure Gene extraction kit (Genta, Redmond, Calif.) to extract DNA from whole blood. PCR for 5-HTTLPR was carried out in a 10 μl volume containing 50 ng of genomic template; 0.5 μM of each primer, one of which was 5' fluorescently labelled; 200 μM of each dNTP; 1 PCRbuffer; 1.5 mmoL/L MgCl2; and 0.3 units of DyNAzymeTM EXT DNA polymerase (Finnzymes Oy, Espoo, Finland), with 0.5 M GCMelt (Clontech, Palo Alto, Calif.). Primer sequences were 5'-FAM-CTGATGCCACCATCCTGATGT3' and 5' GGTTCTGGAATACTGTTAGGCGAAGGACAA3'. Samples were amplified on a 9700 thermal cycler (Applied Biosystems, Foster City, Calif.), with an initial heat-activation step at 96°C for 12 minutes, followed by 40 to 45 cycles, with a denaturation step of 96°C for 30 seconds; an annealing step of 68°C for 45 seconds; and an extension step of 72°C for 3 minutes for 5-HTTLPR, with a final extension step at 72°C for 10 minutes. Post-PCR products were injected and detected by laser-induced fluorescence on an ABI PRISM 3730 Genetic Analyzer (Dallas, TX) at the University of Chicago DNA sequencing and genotyping core. Electropherograms were processed, and alleles were called with Genemapper software blind to platelet data.

Results

The changes in SERT IR induced by fluoxetine exposure were consistent within an experiment (see Fig. 3) and replicable over time in individuals (as shown in Fig. 4). Genotyping of each subject for the SERT promoter polymorphism found that, of the 21 individuals, 5 were homozygous for the long gene (LL), 10 were heterozygous (SL) and 6 were homozygous for the short gene (SS).

An initial inspection of the data was performed to determine whether age, sex or genotype affected fluoxetine-induced change in SERT IR. It was hypothesized that only genotype would have an effect. As the analyses below indi-
cate, age and sex and their interactions with genotype were not statistically significant, whereas genotype alone was a significant factor.

Subjects’ mean age was 32 years (standard deviation [SD] 9.6 yr), which did not correlate with fluoxetine-induced change in SERT IR ($r^2 = 0.01$, df = 1.19, $p = 0.97$). Age did not vary between the SERT genotype groups; people with the SS gene averaged 33 (SD 4) years of age, the SL gene averaged 32 (SD 4) years of age and the LL gene averaged 29 (SD 5) years of age; these were not significantly different (1-way ANOVA, $F_{2,19} = 0.331, p = 0.72$).

Platelets from 3 females (one from each of the LL, SL and SS genotype groups) displayed, on average, a 9% (SD 11%) increase in SERT IR, whereas those from males showed a 0.5% (SD 36%) decrease, which was not significantly different ($t_{20} = 0.344, p = 0.43$). Age did not vary between the SERT genotype groups: people with the SS gene averaged 33 (SD 4) years of age, the SL gene averaged 32 (SD 4) years of age and the LL gene averaged 29 (SD 5) years of age; these were not significantly different (1-way ANOVA, $F_{2,19} = 0.331, p = 0.72$).

There was an effect of SERT genotype on the response to fluoxetine treatment (Fig. 5), which was significant by 1-way ANOVA ($F_{2,19} = 3.582, p < 0.05$). Individuals with the LL genotype were significantly different from those with the SS genotype (Tukey’s $q = 3.705, p < 0.05$), but people with the LS genotype were not significantly different from either homozygous group. On average, those with the SS genotype displayed approximately a 21% (SD 10%) decrease in the 78 kDa SERT IR band, whereas those with the LL genotype displayed a 35 (SD 21%) increase.

Overall, there was no effect of fluoxetine treatment on SERT protein levels, at 38.3 µg/mL (SD 2.1) versus 39.4 mg/mL (SD 2.2) ($t_{20} = 0.1367, 2$-tailed, $p = 0.83$) and no difference on total protein, at 393 mg/mL (SD 41) versus 406 (SD 43) ($t_{20} = 0.1324, 2$-tailed, $p = 0.87$).

Discussion

Understanding the changes induced in SERT function by sustained antidepressant inhibition is important, because an individual’s response to long-term uptake inhibition could be more important than immediate reuptake blockade in explaining eventual clinical response. In the present experiment, exposure to a high dose of the antidepressant drug fluoxetine altered platelet SERT IR in some people. Although there was considerable variation between individuals, the SERT IR response was replicable. Most significantly, the changes induced in SERT IR were related to an individual’s SERT promoter genotype. The SERT promoter genotype is a genetic polymorphism that has been implicated in controlling antidepressant treatment response in several earlier studies and possibly in contributing to depressive symptoms.

In the current experiment, individuals with the SS genotype displayed reduced SERT IR in their platelets after exposure to fluoxetine, compared with vehicle-treated platelets. This suggests that, if parallel effects occur in the human brain, people with the SS genotype might display greater antidepressant effects—both therapeutic and adverse. The existing clinical literature consistently suggests that greater side effects occur in people with the SS genotype, since they are more likely to experience antidepressant-induced mania\textsuperscript{25} and antidepressant-induced insomnia and agitation.\textsuperscript{26} This is easy to conceptualize, because emergence of these side effects would seem more likely in people who display basally lower SERT levels\textsuperscript{4–9} and in those who further undergo an antidepressant-induced decline in SERT function, as suggested in the current study. In addition, other studies indicate that people with the SS genotype are more prone to rapid cycling,\textsuperscript{27} violence\textsuperscript{28} or violent suicide\textsuperscript{29} adverse outcomes that are highly pertinent to antidepressant administration. Thus, further work is needed to confirm whether people with the SS genotype are more prone to these undesirable side effects and whether these are related to a down-regulation of overall SERT concentrations.

In contrast, the preponderance of existing clinical treatment trials indicates that individuals with the SS genotype have poorer clinical response to longer-term antidepressant treatment.\textsuperscript{13–19} (However, there are exceptions: opposite re-
results were noted in 2 of 3 Asian samples evaluated, whereas Yu and others reported results in Han Chinese similar to the European samples.) These results and our own data are less easily explained. Perhaps increased side effects contribute to greater dropouts or mask improvement. Most likely, other unidentified compensatory effects are initiated, nullifying or inducing resistance to the therapeutic neurochemical effects. The decline in SERT IR seen in the platelets from individuals with the SS genotype could reflect degradative or nondestructive, conformational, oligomerization or complexation effects. Further, because the direction of change in SERT IR concentrations appeared to be opposite in people with the LL genotype, our data indicate that multiple distinct processes are likely contributing factors in individuals with each genotype.

The present results, although consistent and suggestive, are preliminary and were discovered in healthy individuals. Similar studies need to be performed in patients with depressive illness. This task is complicated by the fact that it is uncertain whether SERT function is altered or not by depressive illness before beginning antidepressant treatment. If SERT function is previously disturbed in patients with depression, this could obviously confound interpretation of the type of effect presently described in healthy control subjects.

Therapeutic effects are commonly considered slow to evolve, although 24 hours is a short time interval for modeling neuronal response to fluoxetine. However, total platelet half-life is only 10 days, and platelets have limited capacities for more modulated biochemical responses. It is possible that the effects seen in the present experiment after 24 hours might be magnified over time in platelets in vivo or in another model. Conversely, recent data, likely reflecting short-term increases in serotonin levels rather than more complex adaptations, indicate that clinical effects of antidepressant drugs can be detected earlier than previously thought.

It remains to be determined whether the change in platelet SERT IR that we detected was related to a change in SERT function. Although likely, we did not confirm this association in the present study, owing to difficulties in consistently removing inhibitory amounts of fluoxetine. The relation between IR and function is not necessarily a direct one; most transporter molecules are held in reserve and are not functionally active in the outer membrane. Thus, individual differences in the regulation of SERT function may result from genetic variance affecting its trafficking,

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**Fig. 4:** Effects of repeated fluoxetine treatment (0.1 μM for 24 hours) on platelet serotonin transporter (SERT) immunoreactivity (IR) in the same people and replicability of fluoxetine effect on platelet SERT IR. Samples from 5 subjects were assayed at 2 or 3 time points, separated by 2 to 12 months. Each shape represents a unique individual, and the colours represent different genotypes. Black = subjects with an SS genotype, grey = subjects with an SL genotype, and white = subjects with the LL genotype. As shown in the graph, the changes induced were consistent within and between individuals. Each data point represents the results from 3 to 4 sets of paired lanes.

LL = homozygous with the long gene; SL = heterozygous; SS = homozygous with the short gene.

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**Fig. 5:** Effect of fluoxetine treatment (0.1 μM for 24 hours) on platelet serotonin transporter (SERT) immunoreactivity in subjects grouped by SERT genotype. SERT genotype had a significant effect on the response to fluoxetine exposure (1-way analysis of variance; F2,18 = 3.582, p < 0.05). People who were homozygous with the long gene (LL) were significantly different from those homozygous with the short gene (SS) (Tukey’s q = 3.705, p < 0.05).

LS = people with heterozygous genotypes.
including the conformational structure of the SERT protein and interactions with phosphorylative and coupling enzymes that are responsible for recognizing and responding to SERT recycling signals. 3

Despite the fact that platelets and brain neurons are highly distinct physiologically, binding site occupancy-induced molecular changes are likely similar in all membrane-embedded SERT molecules. Because the likely mechanisms do not appear to necessarily invoke complex regulatory loops, further experiments in platelets, and possibly dispersed cells, may illuminate the relevant cell physiology. Also, unlike studies of platelet 5-HT concentrations, the present technique avoids the possible confounding effect of variable 5-HT degradation, which is perturbed by fluctuations in intrinsic monoamine oxidase activity and by potential artifactual oxidation during harvesting or assay. 29 Another strength of our study is that we avoided possible differences in platelet survival or the confounding effects of other internal influences by removing the platelets from the serum environment and controlling the drug exposure in vitro. We believe that further work is necessary to test the hypotheses that fluoxetine exposure alters SERT levels, function and clinical response to antidepressant drugs in platelets and in the brain in a manner related to SS or LL genotype.

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