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The Promiscuity of the Dopamine Transporter: Implications for the Kinetic Analysis of [³H]Serotonin Uptake in Rat Hippocampal and Striatal Synaptosomes

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Summary

Evidence indicates that monoaminergic neurotransmitter transporters are promiscuous, transporting substrates other than their cognate neurotransmitters. For example, serotonin is transported by the dopamine transporter (DAT) under conditions in which serotonin transporter (SERT) activity is eliminated (e.g., pharmacological inhibition). We performed a kinetic analysis of [³H]serotonin uptake in rat striatal synaptosomes (expressing DAT and SERT) and hippocampal synaptosomes (expressing SERT, but not DAT). Nonspecific [³H]serotonin uptake was defined as the amount of uptake remaining in the presence of fluoxetine (10 μ M) or paroxetine (0.05 μ M). In hippocampal synaptosomes, K_m and V_{max} values for [³H]serotonin uptake did not differ whether fluoxetine or paroxetine was used to define nonspecific uptake. However, in striatal synaptosomes, both K_m and V_{max} values for [³H]serotonin uptake were greater when fluoxetine, rather than paroxetine, was used to define nonspecific uptake. These data suggest that, at the concentrations employed, fluoxetine inhibits serotonin uptake at both DAT and SERT, whereas paroxetine only inhibits serotonin uptake at SERT. Thus, when DAT is inhibited by GBR 12909, kinetic parameters for serotonin uptake via SERT in striatum are not different from those obtained in hippocampus. These findings have important implications regarding the analysis of monoaminergic reuptake in brain regions exhibiting heterogeneous transporter expression.

Keywords

neurotransmitter transporter; reuptake; kinetics; dopamine; serotonin

1. Introduction

An accumulating body of literature has demonstrated that monoaminergic neurotransmission occurs through both classical one-to-one synaptic mechanisms and extrasynaptic, volume

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transmission (for review see Zoli et al., 1999). In addition, immunocytochemical evidence reveals that monoamine transporters are located perisynaptically on nerve terminals and along axonal membranes (Pickel et al., 1996, Hersch et al., 1997, Zhou et al., 1998, Tao-Cheng and Zhou, 1999); both the nature of volume transmission and the extra-synaptic localization of monoamine transporters underlies the transport of monoamines between neuronal populations through multiple transporter types. For example, serotonin (5-HT) is readily transported, under certain conditions, into dopamine (DA) neurons in brain regions expressing transporters for both the dopamine transporter (DAT) and serotonin transporter (SERT) (Stamford et al., 1990, Jackson and Wightman, 1995, Zhou et al., 2002, Callaghan et al., 2005, Zhou et al., 2005). Recently, exogenous 5-HT was shown to be cleared from striatum at a faster rate than from hippocampus following a pressure injection of equivalent signal amplitude, as indicated by electrochemical detection, into both brain regions (Callaghan et al., 2005). Results of the latter study demonstrated that the increased striatal 5-HT clearance rate was due to significant 5-HT uptake through DAT as this effect was blocked by application of a DAT inhibitor, GBR 12909. In another study, Zhou and colleagues (2005) showed that striatal dopaminergic terminals will take up 5-HT and subsequently release both 5-HT and DA when SERT is inhibited by a serotonin specific reuptake inhibitor (SSRI) and extracellular levels of 5-HT are elevated. In addition, 5-HT was found to be taken up, stored, and subsequently released by catecholaminergic neurons in rabbit olfactory tubercle under conditions in which serotonin transporter (SERT) activity was inhibited by the SSRI, citalopram (Suarez-Roca and Cubeddu, 2002). Riddle et al. (2003) extended these findings by showing that ceramide, an agent known to alter the phosphorylation state of transporter proteins, increases 5-HT uptake and reduces DA uptake through DAT. 5-HT immunoreactivity has been demonstrated in DA neurons in the midbrain of both SERT knockout mice and wild-type mice treated with the SSRI, paroxetine. The promiscuity of transporter proteins may have significant clinical implications considering the breadth of psychoactive drugs in which the antagonism of monoaminergic transporter activity serves as a primary mechanism of action (e.g., antidepressants, drugs of abuse).

In pre-clinical pharmacological studies, the promiscuity of transporter proteins for substrate is especially relevant when analyzing and comparing the kinetic parameters of monoamine re-uptake across brain regions. In rat striatum, for example, SERT- and DAT-expressing afferent inputs emerge from the midbrain dorsal raphe nuclei and substantia nigra, respectively (Graybiel and Ragsdale, 1983). Although there is dense serotonergic innervation to rat hippocampus originating in the raphe nuclei, this region is essentially devoid of dopaminergic innervation (Fuxe et al., 1985, Donnan et al., 1989, Mennicken et al., 1992). Although numerous studies have investigated the kinetics of 5-HT re-uptake under varying environmental and pharmacological conditions (O'Reilly and Reith, 1988, Asano et al., 1997, Kokoshka et al., 1998, Wells et al., 1999, Martin et al., 2000, Pollier et al., 2000, Nandi et al., 2004, Nightingale et al., 2005) and in varying rat strains (Martin et al., 2000, Fernandez et al., 2001, Fernandez et al., 2003), few studies have considered the impact of the expression of both DAT and SERT in brain regions such as striatum and the potential contribution of DAT to the analysis of 5-HT uptake kinetics. The heterogeneous expression of DAT and SERT in specific brain regions is also an important factor with regard to selecting specific transporter inhibitors and concentrations to define nonspecific uptake. The use of a highly selective inhibitor at a concentration sufficient to saturate the transporter of interest, without inhibiting other monoaminergic transporters, is desirable, yet can be difficult to obtain given the lack of inhibitors demonstrating absolute selectivity.

In the present study, the kinetic parameters (K_m and V_{max}) of 5-HT uptake were determined in rat hippocampal and striatal synaptosomal preparations. To evaluate the contribution of DAT to [3 H]5-HT uptake in striatum, synaptosomes were incubated in the absence or presence of the selective DAT inhibitor GBR 12909 (Rothman et al., 1989). Nonspecific [3 H]5-HT uptake

in both hippocampal and striatal synaptosomes was defined as the amount of uptake remaining in the presence of either fluoxetine or paroxetine.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley rats (225-250g, Harlan Laboratories, Indianapolis, IN) were housed 2 per cage with food and water available *ad libitum*. Rats were maintained under temperature- and humidity-controlled conditions on a 12-hr/12-hr light/dark cycle in the Division of Laboratory Animal Resources in the College of Pharmacy at the University of Kentucky. All experimental procedures were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and approved by the Institutional Animal Care and Use Committee at the University of Kentucky. All efforts were made to minimize animal suffering and to reduce the number of animals used. There were no available alternatives to using rat brain preparations for these *in vitro* analyses.

2.2. Drugs and Chemicals

[³H]5-HT [5-[1,2-³H(N)-hydroxytryptamine creatinine sulfate] (specific activity, 27.1 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). 5-Hydroxytryptamine creatinine sulfate (5-HT), 1-(2-*bis*(4-fluorophenyl)-methoxy)-ethyl-4-(3-phenyl-propyl) piperazine (GBR 12909) HCl, fluoxetine HCl, pargyline HCl, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin, catechol, and L-ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO). α -D-Glucose was purchased from Aldrich Chemical Co. (Milwaukee, WI). Paroxetine HCl was generously provided by Beecham Pharmaceuticals (Surrey, UK).

2.3. Synaptosomal Preparation and [³H]5-HT Uptake

Synaptosomes were prepared from hippocampus or striatum obtained from different rats. For the kinetic analysis of [³H]5-HT uptake into hippocampal synaptosomes, in which there is no DAT expression (Swanson et al., 1987), two sets of experiments were performed in which either: (1) fluoxetine or (2) paroxetine was used to define nonspecific [³H]5-HT uptake. For the kinetic analysis of [³H]5-HT uptake into striatal synaptosomes, in which there is significant DAT expression, four sets of experiments were performed in which synaptosomes were incubated with: (1) fluoxetine to define nonspecific [³H]5-HT uptake and GBR 12909 to inhibit DAT function, (2) fluoxetine to define nonspecific uptake without DAT inhibition, (3) paroxetine to define nonspecific uptake and GBR 12909 to inhibit DAT, or (4) paroxetine to define nonspecific uptake without DAT inhibition. A concentration of 10 μ M fluoxetine was selected based on its common use in kinetics studies of 5-HT uptake inhibition (Asano et al., 1997, Martin et al., 2000, Pollier et al., 2000, Zhang et al., 2002, Fernandez et al., 2003). In an effort to achieve selective 5-HT uptake at SERT, a concentration of 0.05 μ M paroxetine was selected, which is 200-fold higher than its K_i at SERT (0.1 – 0.4 nM; for review see Nemeroff & Owens, 2003) and about 20-fold lower than its K_i at DAT (1.0 μ M; Nemeroff & Owens, 2003; Owens et al., 2001, Koch et al., 2002). Kinetic analyses inform mechanistic evaluations of pharmacotherapeutic candidates as well as current pharmacotherapies. As indicated above, the concentrations employed in these *in vitro* mechanistic analyses, particularly with respect to those chosen to define nonspecific uptake, are based on *in vitro* studies and do not have direct clinical relevance to therapeutic concentrations determined in clinical studies. For example, 10 μ M fluoxetine is an order of magnitude greater than the estimated brain free drug concentration of fluoxetine, and 0.05 μ M paroxetine is close to the minimum estimated brain free drug concentration (Zhou et al., 2007).

Rats were killed by rapid decapitation, brains were removed, and hippocampi or striata were quickly dissected on ice. Hippocampi or striata were homogenized in 20 mL ice-cold sucrose solution (0.32 M sucrose, 5 mM NaHCO₃, pH 7.4) using a Teflon pestle homogenizer (clearance 0.003 inches). Homogenates were then centrifuged (2,000g, 10 min., 4°C) and resulting supernatants (S1) then centrifuged (20,000g, 17 min., 4°C). Pellets (P2) were then resuspended in 2.4 mL of ice-cold Krebs' buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO₄, 1.25 mM CaCl₂, 1.5 mM KH₂PO₄, 10 mM α -D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline, and 0.1 mM ascorbic acid, saturated with 95% O₂/5% CO₂, pH 7.4). Uptake assays were performed in duplicate in a final volume of 500 μ L. Synaptosomes were added in 25 μ L aliquot parts (final protein concentration was 800 μ g/mL) to 375 μ L (or 325 μ L for tubes receiving 2 drugs) Krebs' incubation buffer followed by 50 μ L of drug (fluoxetine, final concentration, 10 μ M; paroxetine, final concentration, 0.05 μ M; and/or GBR 12909, final concentration, 1 μ M). The concentration of GBR 12909 was based on competitive inhibition studies of [³H]DA uptake into striatal synaptosomes (Rothman et al., 1994). After the addition of drugs, assay tubes were then incubated at 34°C for 5 min. Following this initial incubation, 50 μ L of one of nine 5-HT concentrations (1-300 nM; similar to previous studies; e.g., Martin et al., 2000; Pollier et al., 2000; Fernandez et al., 2003) was added to each tube. For these saturation analyses, the final 5-HT concentration was obtained by adding 20 μ L of [³H]5-HT (17 nM) to 30 μ L of cold 5-HT (0.225 – 4.72 μ M). Samples were then incubated at 34°C for 10 min. [³H]5-HT uptake was terminated by the addition of 3 mL ice-cold Krebs' buffer containing catechol (1 mM) and rapid filtration through Whatman GF/B glass fiber filters presoaked in Krebs' buffer containing catechol (1 mM). Filters were washed 3 times with ice-cold buffer, placed in 20 mL scintillation vials containing 10 mL scintillation cocktail, and radioactivity was measured using liquid scintillation spectroscopy (model B1600 TR scintillation counter, Packard Instrument Company, Inc., Downers' Grove, IL). Protein concentrations were determined using the method of Bradford with bovine serum albumin as the standard (Bradford, 1976).

For competition assays, striatal and hippocampal synaptosomes were utilized to determine [³H]DA and [³H]5-HT uptake, respectively. Synaptosomes were prepared using the method described previously. Assays were performed in duplicate in total volume of 500 μ L. Aliquot parts of synaptosomal suspension (50 μ L) were added to tubes containing 350 μ L of Krebs' buffer and 50 μ L of buffer containing 1 nM to 1 mM drug (final concentrations) or 50 μ L of buffer in the absence of drug (control) and incubated at 34°C for 10 min. Following incubation, 50 μ L of [³H]DA (final concentration, 100 nM) or 50 μ L of [³H]5-HT (final concentration, 100 nM) were added to the assay tubes and incubated at 34°C for 10 min. Reactions were terminated by the addition of 3 mL of ice-cold Krebs buffer containing catechol (1 mM). Nonspecific [³H]DA and [³H]5-HT uptake were defined in the presence of 10 μ M GBR 12909 and 10 μ M fluoxetine, respectively. Samples were processed as described above for saturation assays.

2.4. Data Analysis

All values are expressed as mean $\pm$ standard error of the mean (SEM). The mean K_m and V_{max} values (expressed in units of nM and pmol/mg/min, respectively) were calculated from the individual K_m and V_{max} values derived from each experiment using nonlinear regression analysis and a single-site model (GraphPad Prism version 3.0; San Diego, CA). Also, IC₅₀ values from competition assays were generated from nonlinear regression analyses (GraphPad Prism). Resulting K_i values were calculated from IC₅₀ values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Data were analyzed by two-way analysis of variance (ANOVA) followed by one-way ANOVAs within each level of the two between-group variables (DAT inhibitor and SERT inhibitor). Since K_m values are not normally distributed, K_m values were log transformed for parametric statistical analysis. In order to control for family-wise error rate, statistical significance was set at $p < 0.01$.

3. Results

3.1. [³H]5-HT uptake into hippocampal synaptosomes

The kinetics of [³H]5-HT uptake into rat hippocampal synaptosomes was analyzed using fluoxetine (10 μM) or paroxetine (0.05 μM) to define nonspecific [³H]5-HT uptake. As shown in Table 1, the K_m for [³H]5-HT uptake into hippocampal synaptosomes in which fluoxetine was used to define nonspecific uptake was 6.70 ± 1.80 nM with a V_{max} of 0.66 ± 0.04 pmol/mg/min. When paroxetine was used to determine nonspecific uptake, the K_m value was 5.70 ± 1.80 nM and the V_{max} was 0.76 ± 0.11 pmol/mg/min. One-way ANOVA with 5-HT uptake inhibitor as a between-subjects factor demonstrated no significant difference in K_m or V_{max} whether fluoxetine or paroxetine was used to define nonspecific [³H]5-HT uptake ($F_{1,4} = 0.22$, $p = 0.67$ and $F_{1,4} = 0.85$, $p = 0.41$, for K_m and V_{max} , respectively).

3.2. [³H]5-HT uptake into striatal synaptosomes

Striatum was selected as a representative brain region with a heterogeneous monoamine transporter expression to demonstrate the importance of isolating the specific transporter of interest. Striatal synaptosomes were incubated in the absence or presence of GBR 12909 to inhibit uptake through DAT and in the absence or presence of fluoxetine (10 μM) or paroxetine (0.05 μM) to define nonspecific [³H]5-HT uptake. In the absence of GBR 12909 and with fluoxetine used to define nonspecific uptake, the K_m for [³H]5-HT uptake into striatal synaptosomes was 111 ± 4 nM with a V_{max} of 7.80 ± 0.66 pmol/mg/min (Table 1). In the absence of GBR 12909 and with paroxetine used to define nonspecific uptake, the K_m was 8.00 ± 1.52 nM and the V_{max} was 1.52 ± 0.13 pmol/mg/min. Two-way ANOVA with DAT inhibitor (absence vs. presence of GBR 12909) and SERT inhibitor (fluoxetine vs. paroxetine) as between-groups factors revealed a significant interaction between these factors for both K_m and V_{max} ($F_{1,12} = 30.4$, $p < 0.001$ and $F_{1,12} = 21.9$, $p = 0.001$, respectively). Two-way interactions were followed by one-way ANOVAs within the DAT inhibitor and within the SERT inhibitor groups. In striatal synaptosomes incubated in the absence of GBR 12909 (no DAT inhibition), the values for K_m and V_{max} for [³H]5-HT uptake were significantly greater when fluoxetine was used to define nonspecific uptake compared to preparations in which paroxetine was used ($F_{1,9} = 214.36$, $p < 0.001$ and $F_{1,9} = 99.64$, $p < 0.001$, respectively).

In the presence of GBR 12909, the K_m for [³H]5-HT uptake was 18.3 ± 5.36 nM with a V_{max} of 1.44 ± 0.08 pmol/mg/min when fluoxetine was used to define nonspecific uptake (Table 1), and K_m was 8.00 ± 2.00 nM with a V_{max} value of 1.41 ± 0.13 pmol/mg/min using paroxetine to define nonspecific binding (Table 1). Therefore, in the presence of the DAT inhibitor GBR 12909, K_m and V_{max} did not significantly differ between preparations in which either 10 μM fluoxetine or 0.05 μM paroxetine was used to define nonspecific uptake ($F_{1,5} = 5.76$, $p = 0.07$ and $F_{1,5} = 0.03$, $p = 0.87$, respectively). When comparing 5-HT uptake kinetics in which fluoxetine was used to define nonspecific uptake, the K_m and V_{max} were significantly higher in preparations incubated in the absence of GBR 12909 compared to those containing the DAT inhibitor (V_{max} , 7.80 vs. 1.44 pmol/mg/min; K_m , 111 vs. 18 nM; $F_{1,9} = 72.98$, $p < 0.001$ and $F_{1,9} = 36.67$, $p < 0.001$, respectively, see Table 1). However, there were no significant differences in K_m or V_{max} in preparations in which paroxetine was used to define nonspecific uptake regardless of whether GBR 12909 was present ($F_{1,5} = 0.00$, $p = 1.00$ and $F_{1,5} = 0.36$, $p = 0.58$). When paroxetine was used to define nonspecific uptake, the contribution of DAT to total 5-HT uptake was either inhibited by GBR 12909 or subtracted as nonspecific uptake since paroxetine at a concentration of 0.05 μM, specifically inhibits SERT, without inhibiting DAT function.

In a follow-up study to the kinetic analysis of [³H]5-HT uptake in hippocampus and striatum, competition studies were performed and K_i values determined for fluoxetine inhibition of

[³H]DA uptake via DAT in striatal synaptosomes ($K_i = 6.23 \pm 0.49 \mu\text{M}$) and for GBR 12909 inhibition of [³H]5-HT uptake via SERT in hippocampal synaptosomes ($K_i = 0.21 \pm 0.01 \mu\text{M}$).

To demonstrate the effect of the fluoxetine concentration used to define nonspecific [³H]5-HT uptake, a second follow-up study was performed in striatal synaptosomes in the absence of GBR 12909 (no DAT inhibition) and with 1 μM fluoxetine used to define nonspecific uptake. This follow-up study yielded a K_m value of $49 \pm 13 \text{ nM}$ and a V_{max} value of $2.98 \pm 0.55 \text{ pmol/min/mg}$ (See Table 1). The K_m value (49 nM) obtained with 1 μM fluoxetine used to define nonspecific uptake in striatal synaptosomes was significantly different from K_m values obtained with either 10 μM fluoxetine (111 nM) or 0.05 μM paroxetine (8.00 nM) to define nonspecific uptake (one way ANOVA, $F(4,21) = 23.24$, $p < 0.001$; Bonferroni post-hoc tests, 1 μM fluoxetine vs 10 μM fluoxetine, $p = 0.022$; 1 μM fluoxetine vs 0.05 μM paroxetine, $p = 0.003$). The V_{max} value (2.98 pmol/min/mg) obtained with 1 μM fluoxetine used to define nonspecific uptake in striatal synaptosomes was significantly different from the V_{max} value obtained with 10 μM fluoxetine (7.80 pmol/min/mg; one way ANOVA, $F(4,21) = 25.33$, $p < 0.001$, Bonferroni post hoc test: $p < 0.001$,) but not significantly different from the V_{max} value (1.52 pmol/min/mg) obtained with 0.05 μM paroxetine used to define nonspecific uptake.

4. Discussion

The results of the present study demonstrate that the kinetic parameters (K_m and V_{max}) of [³H] 5-HT uptake in hippocampal and striatal synaptosomes do not differ significantly under experimental conditions in which striatal DAT function is inhibited. While numerous studies have analyzed 5-HT uptake kinetics across brain regions, the experimental procedures may not have been optimally suited for isolating SERT activity, most notably in regions expressing both SERT and DAT.

In the present study, the amount of nonspecific 5-HT uptake into hippocampal synaptosomes was defined by incubating samples with either 10 μM fluoxetine or 0.05 μM paroxetine, and measuring the amount of [³H]5-HT remaining. Hippocampus is devoid of significant DAT expression (Swanson et al., 1987) and, as such, it is acceptable to use either fluoxetine or paroxetine over a wide range of concentrations since there is no possibility of higher concentrations inhibiting DAT, as occurs in striatum. When 5-HT uptake kinetics were analyzed in striatum with 10 μM fluoxetine used to define nonspecific uptake, however, the values for K_m and V_{max} were significantly greater compared to values obtained in hippocampal synaptosomes. These results raise two important issues to consider regarding the experimental conditions. First, the increased values for K_m and V_{max} in striatal versus hippocampal synaptosomes appear to be due to significant 5-HT uptake *via* DAT. This is not surprising considering that striatal neurons establish synaptic contact with both DAT- and SERT-expressing terminals (Graybiel and Ragsdale, 1983) and that 5-HT can be transported as a substrate for DAT and modulate DAT activity (Stamford et al., 1990, Jacocks and Cox, 1992, Jackson and Wightman, 1995, De Deurwaerdere et al., 1996, Suarez-Roca and Cubeddu, 2002, Zhou et al., 2002, Riddle et al., 2003, Callaghan et al., 2005). Second, the concentration of fluoxetine (10 μM) used for defining nonspecific [³H]5-HT uptake in striatal synaptosomes appears to have inhibited DAT. Fluoxetine has been shown to inhibit [³H]DA uptake and [¹²⁵I]-RTI-55 binding to DAT with a K_i of 3-10 μM (Owens et al., 2001, Koch et al., 2002). In a follow-up series of competition assays, a K_i value of 6.23 μM for fluoxetine inhibition of [³H]DA via DAT in striatal synaptosomes was obtained, consistent with previous reports. Thus, under certain experimental conditions including: (1) failure to inhibit DAT or other neighboring transporters capable of transporting [³H]5-HT as a substrate and (2) relatively high concentrations of [³H]5-HT (1-300 nM), [³H]5-HT uptake through DAT may be incorrectly calculated as part of specific uptake when determining K_m and V_{max} values for SERT. This

appears to be the case in prior kinetic analyses using a similar concentration range of [^3H]5-HT.

To demonstrate this phenomenon, a second series of experiments was performed in which DAT activity was inhibited with GBR 12909 (1 μM) and nonspecific [^3H]5-HT uptake was again defined by 10 μM fluoxetine or 0.05 μM paroxetine, a selective SERT inhibitor with higher affinity for SERT than fluoxetine, and measuring the amount of [^3H]5-HT transported. This concentration of paroxetine was expected to specifically inhibit SERT, while producing negligible inhibition of DAT activity as previously reported (Owens et al., 2001; Koch et al., 2002). In the presence of GBR 12909, the K_m and V_{max} for [^3H]5-HT uptake were not significantly different between striatal synaptosomes in which nonspecific uptake was defined with 10 μM fluoxetine or 0.05 μM paroxetine. Under these conditions, the SERT inhibitor and concentration used to determine nonspecific [^3H]5-HT uptake is less important, because extracellular 5-HT will not be taken up by DAT. The K_m and V_{max} for 5-HT uptake also did not differ significantly in striatal synaptosomes in which nonspecific uptake was defined in the presence of 0.05 μM paroxetine, regardless of whether or not GBR 12909 was included in the incubation buffer. The kinetic parameters did not differ in the presence of paroxetine with or without GBR 12909, because in each of these conditions, 5-HT uptake through DAT was either inhibited by GBR 12909 or subtracted as nonspecific uptake.

Importantly, the concentration of GBR 12909 (1 μM) used to inhibit DAT in striatum in the current study was based on numerous competition assays in studies of [^3H]DA uptake into striatal synaptosomes (e.g., Rothman et al., 1994), and this concentration is 1000-fold higher than the reported K_i value for inhibition of [^3H]DA uptake into striatal synaptosomes (e.g., Matecka et al., 1997). Current follow-up experiments determined a K_i value of 0.21 ± 0.01 μM for GBR 12909 inhibition of [^3H]5-HT uptake into hippocampal synaptosomes (data not shown), suggesting that the concentration of GBR 12909 used to inhibit DAT in striatal synaptosomes may have also affected 5-HT uptake via SERT. However, the presence of GBR 12909 did not affect the V_{max} values obtained in striatal synaptosomes incubated with paroxetine, whereas a marked decrease in V_{max} was expected if GBR 12909 was inhibiting a significant number of SERT sites.

In general, the K_m and V_{max} values reported here for 5-HT uptake in hippocampal and striatal synaptosomes are lower than those previously reported in the literature. This disparity may be the result of differences in species, rat strain, or synaptosomal preparation procedures. The difference in rat strain may not be trivial considering that Martin and colleagues (2000) found a two-fold difference in K_m and V_{max} values for [^3H]5-HT uptake into hippocampal synaptosomes in SHR rats compared to Lewis rats. In the present study, K_m and V_{max} values in hippocampus were an order of magnitude lower than those reported by Martin et al. (2000). This difference may reflect the current use of Sprague-Dawley rats and an incubation time of 10 minutes compared to 5 minutes in the Martin study. The apparent disparity between our findings and those of previous studies may also be the result of 5-HT uptake *via* DAT and SERT; most notably under experimental circumstances in which the contribution of DAT was not inhibited. In striatal synaptosomes incubated without GBR 12909 and using 10 μM fluoxetine to define nonspecific uptake, the K_m value that we report is consistent with those reported in previous studies using striatal synaptosomes without DAT inhibition (Martin et al., 2000; Pollier et al., 2000).

Although the current study only examines 5-HT uptake kinetics in striatal and hippocampal synaptosomes, previous studies have analyzed 5-HT uptake kinetics in midbrain synaptosomal preparations using 10 μM fluoxetine to define nonspecific uptake (Zhang et al., 2002; Fernandez et al., 2003). Zhang and colleagues (2002) report a K_m value for 5-HT uptake in midbrain that is an order of magnitude higher than that currently reported in hippocampus. One

explanation for this disparity may be divergent transporter expression in midbrain as compared to hippocampus, most notably those transporters capable of transporting [^3H]5-HT. The midbrain ventral tegmental area and substantia nigra both display significant DAT expression (Mennicken et al., 1992, Ciliax et al., 1995, Freed et al., 1995, Hersch et al., 1997) and, as such, the reported kinetic analyses of 5-HT uptake into midbrain synaptosomes may reflect uptake via multiple transporters and not uptake specifically via SERT. In a more recent study, Samuvel and colleagues (2005) reported K_m and V_{max} values for 5-HT uptake into midbrain synaptosomes using 0.1 μM fluoxetine to define nonspecific uptake. Values obtained in the latter study are consistent with those currently reported for 5-HT uptake into striatal synaptosomes, using 0.05 μM paroxetine to define nonspecific uptake, a condition which accurately represents 5-HT uptake specifically via SERT. Thus, the Samuvel study (2005) is an excellent example of an uptake experiment in which a serotonin-specific reuptake inhibitor (fluoxetine) was used to define nonspecific uptake at a concentration (0.1 μM) that specifically inhibited SERT and not DAT, in midbrain synaptosomes containing a heterogeneous assembly of monoaminergic transporters.

The results of the current study also raise similar questions with regard to synaptosomal uptake studies in which nonspecific uptake is defined by parallel preparations incubated at 4°C. Transmitter uptake through membrane-bound synaptic transporters is temperature-dependent (at or near 37°C; Kirpekar and Wakade, 1968, Lindmar and Loffelholz, 1972, Vizi, 1998). Thus, incubating synaptosomes on ice is expected to inhibit transporter activity and any substrate accumulated at 4°C is considered nonspecific. Racca et al. (2005) and Inserte et al. (1999) investigated the kinetic parameters of 5-HT uptake into synaptosomal preparations from several brain regions, including DAT-containing areas such as midbrain, frontal cortex and striatum (Inserte et al., 1999, Racca et al., 2005). In each of these studies, nonspecific uptake was defined by measuring the radiolabel taken up in parallel synaptosomal aliquots incubated at 4°C. K_m and V_{max} values reported in these studies may reflect transport through multiple monoaminergic transporter (e.g., 5-HT uptake through both SERT and DAT), because inhibition of transporter activity (both SERT and DAT) via incubation at low temperature would presumably achieve the same result as inhibition of DAT and SERT with 10 μM fluoxetine (current study). Thus, 5-HT uptake by DAT would not be subtracted from total uptake nor be accounted for as nonspecific uptake, and K_m and V_{max} values would reflect uptake erroneously through both DAT and SERT.

In addition to DAT, other transporters that may influence 5-HT uptake kinetics at SERT are important to consider. Although the hippocampus is essentially devoid of DAT expression, this brain region does possess a significant amount of norepinephrine transporter (NET) expression (Schroeter et al., 2000). However, it is unlikely that 5-HT uptake via NET contributed to the currently reported K_m and V_{max} values. The concentrations of paroxetine (0.05 μM) used to define nonspecific 5-HT uptake in hippocampal synaptosomes is 3-fold lower than the K_i reported for paroxetine inhibition of NET activity (Owens et al., 2001). The concentration of fluoxetine (10 μM) used to define nonspecific uptake in hippocampus was equal to or higher than the reported K_i for fluoxetine to inhibit NET (0.6 – 10 μM ; Fuller et al., 1991, Owens et al., 2001), suggesting that 5-HT uptake via NET may have been incorrectly included as specific uptake at SERT. However, no significant differences in K_m or V_{max} values were observed in hippocampal synaptosomes incubated with fluoxetine versus paroxetine to define nonspecific uptake. This may be due to greater SERT expression (and presumably more sites for 5-HT uptake) relative to NET in hippocampus (Daws et al., 1998), such that any contribution of NET to specific uptake is minimal relative to the contribution of uptake via SERT.

A novel plasma membrane monoamine transporter (PMAT) was recently identified by Zhou and others (2007) as an additional mechanism by which 5-HT may be transported from the

extracellular space in several brain regions. The IC_{50} values for fluoxetine and paroxetine inhibition of PMAT has been reported to be 28 and 22 μM , respectively (Zhou et al., 2007). With respect to the result of the present study, the contribution of PMAT and its inhibition by 10 μM fluoxetine in our synaptosomal preparations can not be ruled out; the incorrect inclusion of some 5-HT uptake via PMAT may have been incorporated in the determination of SERT specific 5-HT uptake when fluoxetine was used to define nonspecific uptake. However, using 0.05 μM paroxetine eliminates the potential contribution of PMAT to total [3H]5-HT uptake given the 22 μM K_i value for paroxetine inhibition of PMAT. [3H]5-HT uptake via PMAT would be appropriately subtracted as nonspecific uptake.

The results of the present study demonstrate the importance of considering: (1) the heterogeneity of transporter expression in brain regions of interest and (2) the selection and concentration of transporter inhibitor to be used for defining nonspecific uptake when performing kinetic analyses of transmitter uptake through a specific transporter type. The first series of experiments revealed that for kinetic analyses of 5-HT uptake in striatum, which expresses both SERT and DAT, a concentration of 10 μM fluoxetine was not selective, but found to inhibit DAT function, and thus, was not appropriate for defining nonspecific 5-HT uptake in this heterogeneous brain region. In a second series of experiments, both the selection and concentration of transporter inhibitor used to define nonspecific 5-HT uptake was addressed using a more selective inhibitor paroxetine at a concentration of 0.05 μM that inhibits SERT, but not DAT. Under these conditions, the values obtained for K_m and V_{max} were consistent with those generated in hippocampus, a brain region with high SERT expression and no DAT expression.

In the present study, fluoxetine was found to have a K_i of 6.23 μM for inhibition of [3H]DA uptake via DAT, such that, the use of 1 μM fluoxetine to define nonspecific would be expected to only partially inhibit DAT in striatum. In a third series of experiments, this lower concentration of fluoxetine (1 μM) was used to define nonspecific 5-HT uptake in striatum and the values for K_m (49 nM) and V_{max} (2.98 pmol/min/mg) were intermediate compared to those generated in earlier experiments using 10 μM fluoxetine (111 nM and 7.8 pmol/min/mg, respectively) or 0.05 μM paroxetine (8 nM and 1.41 pmol/min/mg, respectively). Based on these results, we predict that the use of 100 nM fluoxetine to define nonspecific [3H]5-HT uptake in striatum would result in K_m and V_{max} values consistent with those generated from hippocampus and those generated in striatum using 0.05 μM paroxetine. Thus, the permissiveness of DAT to take up 5-HT and the selectivity of the drugs used to define nonspecific should be taken into careful consideration when performing kinetic analyses in heterogeneous brain regions..

Results of the current study have important implications for pre-clinical evaluations of potential antidepressant drugs which often utilize *in vitro* neurotransmitter uptake assays to determine K_m and V_{max} values. Such values must be considered in the context of the brain region evaluated, the transporters expressed in the brain region, and the selection of compounds as transporter inhibitors to define nonspecific uptake. The often disregarded phenomenon of transmitter uptake through multiple transporter types is especially important to consider given the ongoing discovery of novel transporters.

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Abbreviations

5-HT	serotonin
ANOVA	analysis of variance
DA	dopamine
DAT	dopamine transporter

HEPES

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

NET

norepinephrine transporter

SERT

serotonin transporter

Table 1Summary of [³H]-5-HT uptake into hippocampal and striatal synaptosomes

	K_m (nM)	V_{max} (pmol/mg/min)
Hippocampus		
Fluoxetine (10 μ M)	6.70 ± 1.80	0.66 ± 0.04
Paroxetine (0.05 μ M)	5.70 ± 1.80	0.76 ± 0.11
Striatum		
Absence of GBR 12909 (1 μM)		
Fluoxetine (10 μ M)	$111 \pm 10^{* \dagger}$	$7.80 \pm 0.66^{* \dagger}$
Fluoxetine (1 μ M)	$49.0 \pm 13^*$	2.98 ± 0.55
Paroxetine (0.05 μ M)	8.00 ± 1.52	1.52 ± 0.13
Presence of GBR 12909 (1 μM)		
Fluoxetine (10 μ M)	18.3 ± 5.36	1.44 ± 0.08
Paroxetine (0.05 μ M)	8.00 ± 2.00	1.41 ± 0.12

Uptake assays were performed in the presence of nine concentrations of [³H]-5-HT ranging from 0.001-0.3 μ M. Data are expressed as the mean $\pm$ SEM from 3 -7 experiments.

* $p < 0.01$ compared to striatal preparations in which paroxetine was used to calculate nonspecific uptake in the absence of GBR 12909

$\dagger$ $p < 0.01$ compared to striatal preparations in which fluoxetine was used to calculate nonspecific uptake in the presence of GBR 12909.